Construction of a robust phylogeny facilitates development of an environmental DNA-based survey tool for the bog hoverfly, *Eristalis cryptarum*

Volume 1 of 1

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I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

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Thesis Summary

Surveying an endangered species is vital for its successful conservation. The use of environmental DNA (eDNA) as surveying tool for rare and elusive species has gained popularity over recent years. *Eristalis cryptarum,* commonly known as the bog hoverfly is listed as a priority species on the UK Biodiversity Action Plan, is Critically Endangered and restricted to few sites on Dartmoor National Park. It is widely assumed *E. cryptarum* have an aquatic, rat-tailed larval stage, as is the case for other closely related species. The larvae however, have never been discovered in the UK in order to determine this. In Chapter 1, using the mitochondrial gene cytochrome *c* oxidase subunit 1 (*cox1*), the phylogeny of *E. cryptarum* is explored. Using molecular data and morphological characteristics, *E. cryptarum* is placed within the Eristalini tribe, a tribe distinguished by an aquatic, rat-tailed larval stage. This offers further support for the assumption that *E. cryptarum* possesses an aquatic, rat-tailed larval stage. The use of eDNA as a tool to survey endangered species has been widely used in previous studies and offers a sensitive, non-invasive approach to survey elusive and rare species. Here, larval *E. cryptarum* eDNA will be screened for in water samples collected from known habitat sites on Dartmoor National Park. Chapter 2 is focused on the development on taxa-specific primer sets and are tested for specificity and sensitivity in preparation for eDNA screening in Chapter 3. Primer sets were designed and developed successfully with high specificity to target taxa and shown to be sensitive through a number of dilution series. The use of an environmental DNA technique to determine the presence or absence of *E. cryptarum* eDNA in water samples is an exciting alternative to traditional surveying techniques. Chapter 3 explores this, and

there was no amplification of *E. cryptarum* eDNA but a successful amplification of a closely related species, *E. arbustorum* from water samples where *E. arbustorum* was known to be present (using *E. arbustorum* specific primer sets). This suggests the need for further research and optimisation of this method for successful surveying of *E. cryptarum* using an eDNA methodology.

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Table of Contents

List of Tables and Figures

Chapter 1: General Introduction

1.1 The bog hoverfly *Eristalis cryptarum*

Eristalis cryptarum distribution and habitat requirements

The critically endangered bog hoverfly *Eristalis cryptarum* (Fabricuius, 1794) has always been a rare species in the United Kingdom, but historic records are distributed across the majority of southwest England. Up until 1951, *E. cryptarum* localities were scattered in Cornwall, Devon, Dorset heaths, Somerset and the New Forest (Levy & Levy, 1992; Stubbs and Falk, 2002; Ball & Morris, 2014). There is also one ambiguous record from Gloucestershire which remains unconfirmed (Stubbs and Falk, 2002; Ball & Morris, 2014). Since 1951 (where *E. cryptarum* was recorded in the New Forest heathland bogs), sightings have been restricted to the Dartmoor National Park in Devon, Southwest England and two records of *E. cryptarum* in 1978 from Newbridge, Dartmoor were the last recorded sightings of this species. This species was thought to be extinct until its rediscovery on Dartmoor in 1993 (Ball & Morris, 2014). Due to this dramatic decline, *E. cryptarum* is critically endangered and listed as a priority species on the UK Biodiversity Action Plan (UK Biodiversity Group, 1999). As well as this, *E. cryptarum* is a Red Data Book 2 species (Shirt, 1987).

Since the rediscovery of *E. cryptarum* in 1993, this species has only ever been found on Dartmoor National Park. Even so, the distribution of *E. cryptarum* is still limited and restricted to 18 known sites that generally fall in the catchments of the East and West Webburn and West Dart (Drake & Baldock, 2005).

Moortown Bottom, Challacombe and Buckland Common hold the most recent records of this species prior to this project (Table 1).

Table 1 Sites on Dartmoor National Park where *E. cryptarum* was last recorded, including the year and the recorder: Martin Drake (MD), John Walters (JW), Norman Baldock (MB) and Nigel Pinhorn (NP).

Site	Grid reference	Most recent record	Recorder
Smoothmoor	SX 721772	2004	MD
Pizwell	SX 670786	2004	MD
Middle Merripit	SX 647798	2004	NB
Broadaford	SX 689772	2004	MD
Lizwell Mead	SX 696776	2004	NB
Challacombe	SX 694792	2014	MD
Grendon	SX 687783	2004	MD
Corndonford Mire	SX 692748	15.8.2004	JW
Haytor	SX 766771	17.7.2008	NB
Bonehill	SX 733777	16.6.2003	JW
Buckland Common	SX 734744	2014	MD
Huccaby	SX 658732	2002	MD
Sherberton	SX 648732	2004	MD
Moortown Bottom	SX 661893	2014	NB
Chalk Ford	SX 682682	28.5.2003	JW
Lower Hurston	SX 688843	2004	NB
Yellands	SX 689848	2004	MD
Fernworthy Reserve	SX 6635383815	2009	NP via JW

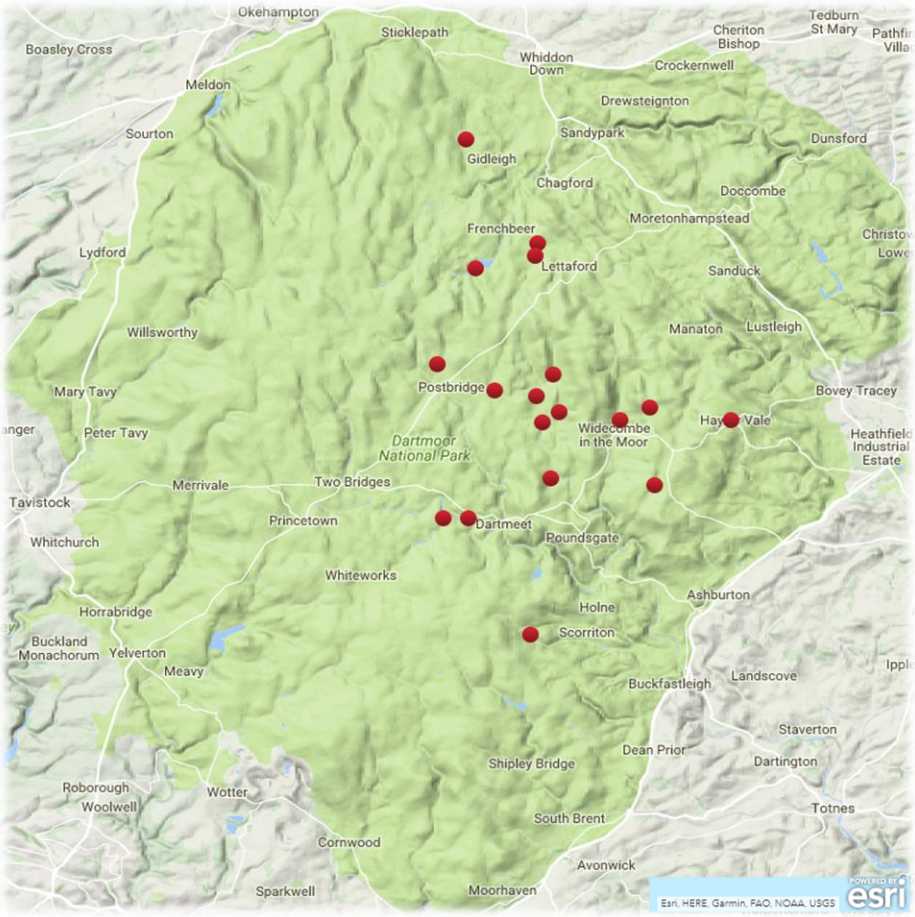


Fig. 1 A map of Dartmoor National Park marked with the sites (Table 1) that hold past records for *E. cryptarum* (ArcGIS Online hosted by Esri).

Understanding the full range of this species on a global scale is difficult. In total, there are 16 records submitted to The Barcode of Life Data system (BOLD) (Ratnasingham & Herbert, 2007) and GenBank combined (with sequence data for the mitochondrial gene cytochrome *c* oxidase subunit 1). These records include information of where *E. cryptarum* specimens were collected (Table 1). Most of these records originate from Canada, Alaska and northern Europe. Nielsen and Svendsen (2014) published hoverfly records that they had collected themselves in north Norway including *E. cryptarum.*

As the common name suggests, the bog hoverfly is found in wet and boggy habitats. Specifically, this species has an association with valley mires within Rhôs pasture systems near the edges of open moors (Drake & Baldock, 2013). Rhôs pastures are areas of marshy grasslands that are moderately grazed by ponies or cattle and are generally dominated by purple moor grass *Molinia* and rushes *Juncus* (Castle & Falk, 2013). These habitats support a variety of wildflowers, some of which are important nectar sources for *E. cryptarum.* These include: bog bean *Menyanthes trifoliata,* marsh marigold *Caltha palustris,* bog asphodel *Narthecium ossifragum,* marsh St John's wort *Hypercium elodes,* marsh lousewort *Pedicularis palustris,* Devils-bit scabious *Succsia pratensis* and heather *Calluna vulgaris* (Ramel, 1998; Drake & Baldock, 2005; Walters, 2008).

Fig. 2 *Eristalis cryptarum* on marsh St John's wort *Hypercium elodes* © John Walters.

E. cryptarum is a very distinctive and attractive species. Even though there are similarities with other smaller *Eristalis* species such as *E. nemorum*, it is readily identifiable by eye in the field, with completely red-orange legs, red hairs covering the thorax and two triangular red spots on its second tergite (Stubbs & Falk, 2002) (Fig. 2). But, despite its distinctive appearance, this species is well

known amongst local naturalists as being very elusive and easily disturbed, the smallest disturbance causing *E. cryptarum* to fly away (Perrett, 2001; Drake & Baldock, 2005).

Reasons for E. cryptarum species decline

Eristalis cryptarum has always been a fairly rare species but has seemed to have severely declined. A clear reason for this decline is not known but there have been various thoughts and ideas as to why this may be. One theory is that *E. cryptarum* is a poor competitor (Castle & Falk, 2012). Other species of the *Eristalis* genus such as, *E. horticola, E. tenax, E. intricarius* and *E. pertinax* are all common species and are found in the same habitat as *E. cryptarum* (but not exclusively, unlike *E. cryptarum*) in high numbers. It is possible that these common and larger species are outcompeting *E. cryptarum* for nectar sources.

Castle and Falk (2012) also discuss the possibility of *E. cryptarum* populations being vulnerable to the presence of a muscid fly *Graphomya maculata,* which are predators of *Eristalis* larvae. Where *E. cryptarum* presence was higher at a particular site, the presence of *G. maculata* seemed to be lower (Castle & Falk, 2012).

Other thoughts as to why *E. cryptarum* may be in decline are the changes in traditional grazing practices at these sites (Castle and Falk, 2012). The maintenance of the habitat of the bog hoverfly requires moderate grazing by livestock and Dartmoor ponies to keep vegetation short, to allow the growth of important wildflowers (Drake & Baldock, 2005) and possibly the presence of fresh dung may be of importance too (Perrett, 2001; Castle and Falk, 2012; Ball and Morris, 2014). Furthermore, grazing also contributes to the maintenance of

willow and birch growth, which if left, this habitat will be vulnerable to scrub invasion (Ball & Morris, 2014). The presence of grazing animals may also be important as the hoof prints left by livestock and Dartmoor ponies may be important in opening the habitat for ovipositing females, as seen in other *Eristalis* species (Drake & Baldock, 2005). The open seepages and runnels characteristic of this habitat is also maintained by the movement of livestock and Dartmoor ponies. This seem to be vital, as males have only ever been seen to display territorial behaviours (that may relate to mating) at the edges of these seepages and runnels (Drake & Baldock, 2005).

Eristalis cryptarum larval stage

The egg and larval stage of *E. cryptarum* is very poorly understood and contributes to a large knowledge gap in the understanding of the life history of *E. cryptarum*. The only reference to the egg stage in the life cycle of the bog hoverfly was by Kutznetsov (1989 & 1992). A scanning electron microscope (SEM) was used to compare the egg morphology of 37 species of hoverfly and a key developed to be used for hoverfly egg identification. The egg of *E. cryptarum* was described in this study for the first time, however the details of where the eggs were found and collected from was not disclosed. The description of the egg is as follows: The eggs were narrow and oblong, measured at 2.01 by 0.55 mm and were white. Other details included: the micropyle (the opening to allow sperm to penetrate for fertilisation) being a short and broad tube with a diameter of 0.01 mm. The chorion ultrastructure (EUS) around the micropyle was short, broad and flattened. The EUS length was 0.02 mm and is in the form of flat and round rings (full definitions of egg

morphological characters are given in this paper). The only reference found in regard to the larvae is also by the same author (Kuznetsov, 1992). Here a description and images are provided for the first instar larvae of *E. cryptarum,* however information regarding where the larvae was collected is not given. To the author's knowledge, there is no documentation of more than one larval instar stage nor the pupae. In the UK there has been no record of *E. cryptarum* larvae. It is widely accepted by naturalists and entomologists that the larvae are aquatic and of the 'rat-tailed' variety, similar to the rest of the *Eristalis* genus and other closely related species (Stubbs & Falk, 2002). The long or 'rat' tail of the larvae is used as a type of snorkel and allows the larvae to live in wet habitats, whether that be submerged in water, in wet mud or with aquatic plants that are partially underwater (Fig. 3). However, this does not necessarily mean that the adults are tied to this type of habitat. In fact, the more common species such as *Eristalis tenax* or *Helophilus pendulus* can be present a far distance from breeding sites (Stubbs & Falk, 2002).

Fig. 3 Rat or long tailed larvae of *E. arbustorum,* typical of the Eristalini tribe.

Previous ecological studies regarding E. cryptarum on Dartmoor

There have been a number of past ecological studies in regard to the survey of *E. cryptarum.* These have all generally been focused on determining the distribution of *E. cryptarum* on Dartmoor, recording the number of individuals observed, what flowers are used by feeding adults, habitat characteristics and the search for *E. cryptarum* larvae.

In 1998, G. Ramel submitted a report to English Nature with his findings. Sites that held historical records were visited and the number of *E. cryptarum* sighted and their sex was documented. Ramel recorded *E. cryptarum* at six sites in total and some key characteristics of the habitat favoured by *E. cryptarum* such as the presence and potential importance of the different species of wildflowers as nectar sources and sphagnum moss blankets *Sphagnum sp.*

Perrett (2001) followed this work by visiting the same sites described by Ramel (1998) to expand the current knowledge of this species habitat requirements, to observe breeding behaviours and oviposition and also with the hope of locating the larvae. An additional aim was to identify new sites to add to the known distribution of the bog hoverfly. An interesting observation as a result of this study was the sighting of a female laying eggs in a freshly laid cow pat, a process which took approximately 3-4 minutes. However, any attempts to rear or find larvae in samples taken from this particular dung pat failed. Another key observation was on two occasions where males were observed to defend a territory along a runnel by flying in to another individual, presumably for breeding purposes. This study really highlighted the difficulty of surveying this species and many ‘reliable’ sites had to be revisited before *E. cryptarum* was even recorded.

Drake and Baldock (2002, 2003 & 2004) carried out a study over three years for the Dartmoor National Park Authority. Here the approach was similar to previous work by Ramel (1998) and Perrett (2001) to record sightings of adults at various sites. In 2002, 17 sites were visited (sites that held historical records of the bog hoverfly and other potential habitats) and observations of adult *E. cryptarum* were recorded, as well as their preferred flowers for feeding as observed at the time. Where possible, individual adult *E. cryptarum* were caught and marked in an attempt to measure the extent of the population using a mark-recapture method. Dung was also collected and placed along runnels in an attempt to attract ovipositing females, as observed by Perrett (2001), however oviposition was not documented again. Samples were also collected from small crevices (for example, hoof prints) and placed in a white tray to search for larvae, which again was unsuccessful. In total *E. cryptarum* was recorded at 6 sites (Challacombe Farm, Lizwell Mead, Pizwell, Great Cator, Lower Hurston and Smoothmoor) during 24th April – 22nd September 2002.

In the second year of this study, there was more of a focus on the marking of individuals to estimate population size. Over a two-week period, 45 individuals were marked, 21 of these were never recaptured. This data was used to estimate the lifespan of *E. cryptarum* of 18.75 days and the population size was estimated at this site to be around 40 flies. This study also looked at the data collected previous to this study and concluded that there seems to be a population peak in late May and a smaller peak in July (Drake & Baldock, 2003, 2002, Ramel, 1998, Perrett, 2001).

The last year of this study (Drake & Baldock, 2004) involved similar methods as in previous years. Larvae were searched for by collecting vegetation along seepages where adults had been recorded in past years. This vegetation was

inspected in laboratories with no success. Challacombe was the site chosen to continue mark-recapture work in order to estimate population size and general monitoring of adults continued using the same method from previous years at known sites or in new places in a search for new *E. cryptarum* habitat. The results obtained from the mark-recapture work gave an insight in to the lifespan of this species, which was estimated to be between 13-25 days (average of 19.5 days), similar to the previous year but in both years the mark and recapture rates were very low. This makes it difficult to infer a concrete conclusion about the population size and life span of this species. As mentioned previously, no larvae were discovered in this year of study, but local naturalist John Walters reported a female ovipositing in peat in late May (Drake & Baldock, 2004).

In 2008, 16 sites known to hold records of *E. cryptarum* were visited (Walters, 2008) but this was a year of poor weather, and so adult *E. cryptarum* was recorded at only 5 of these sites. The bog hoverfly, as previously described is notoriously difficult to survey and this species has only been recorded on days where the conditions are optimal i.e. sunny, little wind and no rain (Drake & Baldock, 2002, 2003, 2004, Perrett, 2001, Castle & Falk, 2012, Ramel, 1998).

A project led by Buglife (Castle & Falk, 2012) set out to the same sites as explored in previous studies to determine the range of the bog hoverfly populations, as well as investigation the larval ecology of this species. 9 individuals were recorded in 3 sites (Pizwell, Challacombe Farm and Buckland Common) all in late August and early September. In contrast to other studies, some adult *E. cryptarum* were caught by sweep netting, but most sightings are of adults basking or feeding and are viewed from a distance using binoculars (Drake & Baldock 2002, 2003, 2004, Walters, 2008, Perret 2001, Ramel, 1998).

These past studies have expanded the knowledge surrounding this endangered species including its feeding habits and distribution on Dartmoor. But they highlight the extreme difficulty in surveying this particularly elusive species and the gap in our knowledge regarding breeding and larval stage is evident.

1.2 Phylogeny of *Eristalis cryptarum*

Syrphidae (Diptera), or what is known commonly as hover or flower flies, are a wonderfully diverse dipteran family, with over 5000 species worldwide, 281 of which are found in the UK (Ball & Morris, 2015). The adults are important pollinators as nearly all uniformly feed on nectar and pollen (Rader *et al.* 2016; Young *et al.* 2016) but the larvae display a vast array of feeding strategies, from phytophagous, mycophages, saprophages and predacious (Rotheray, 1993). Pipunculidae (the big-headed flies) have been shown using molecular data to be a sister family to the Syrphidae to form a superfamily Syrphoidea (Skevington & Yeates, 2000). There are three subfamilies of British hoverflies in the Syrphidae family: Syrphinae, Milesiinae (Eristalinae) and Microdontinae which are further divided in to a total of 14 tribes (Rotheray & Gilbert, 1999; Stubbs & Falk, 2002; Ball & Morris, 2015) (Table 2).

Table 2 The three subfamilies of the British hoverfly family Syrphidae including the tribes and genera.

Subfamily	Tribe	Genus
Syrphinae	Bacchini	*Baccha*
		Melanstoma
		Platycheirus
		Xanthandrus
	Paragini	*Paragus*

Table 2 continued.

Subfamily	Tribe	Genus
		Didea
		Doros
		Epistrophe
		Episyrphus
		Eriozona
		Eupeodes
		Leucozona
		Megasyrphus
		Melangyna
		Meligramma
		Meliscaeva
		Parasyrphus
		Scaeva
		Sphaerophoria
		Syrphus
		Xanthogramma
Milesiinae (Eristalinae)	Callicerini	*Callicera*
	Cheilosiini	*Cheilosia*
		Ferdinandea
		Portevinia
		Rhingia
	Chrysogastrini	*Brachyopa*
		Chrysogaster
		Hammerschmidtia
		Lejogaster
		Melanogaster
		Myolepta
		Neoascia
		Orthonevra
		Riponnensia
		Sphegina
	Eristalini	*Anasimyia*
		Eristalinus
		Eristalis
		Helophilus
		Lejops
		Mallota
		Myathropa
		Parhelophilus
	Merodontini	*Eumerus*
		Merodon
		Psilota
	Pelecocerini	*Chamaesyrphus*
		Pelecocera

Table 2 continued.

Subfamily	Tribe	Genus
	Pipizini	*Heringia*
		Pipiza
		Pipizella
		Trichopsomyia
		Triglyphus
	Sericomyiini	*Arctophila*
		Sericomyia
	Volucellini	*Volucella*
	Xylotini	*Blera*
		Brachypalpoides
		Brachypalpus
		Caliprobola
		Chalcosyrphus
		Criorhina
		Pocota
		Syritta
		Tropidia
		Xylota
Microdontinae		*Microdon*

Morphologically, the Eristalini tribe (subfamily Milesiinae) is one of the most recognisable hoverfly tribes with a strong loop easily visible on the R_{4+5} vein (Fig. 4), a character only otherwise present in *Merodon* and a few other *Syrphus* groups (but combinations of other phenotypic characters support the placement of these species outside of the Eristalini tribe) (Stubbs & Falk, 2002). The larvae are a key characteristic to the Eristalini tribe as they are of the distinctive long or rat-tailed maggot type (Rotheray & Gilbert, 1999). There are 8 genera within the Eristalini tribe: *Myathropa*, *Mallota*, *Helophilus*, *Eristalinus*, *Eristalis*, *Parhelophilus*, *Lejops* and *Anasimyia* (Rotheray & Gilbert, 1999; Stubbs & Falk, 2002; Ball & Stubbs, 2015). Kanervo (1938) split the *Eristalis* genus in to two subgenera: *Eoseristalis* and *Eristalis* using adult male genitalia characteristics. The *Eoseristalis* genus is defined by the basal half of the hind tibia being pale

and they lack obvious hair fringes on the hind tibia (compared to the honey bee mimic *Eristalis tenax,* the only species found in the *Eristalis* subgenus) (Kanervo, 1938; Stubbs & Falk, 2002). However, even though this split is acknowledged in the British identification guide to hoverflies by Stubbs & Falk (2002) and by other authors (for example Thompson, 1997), this division is considered to generally be ignored (Pérex-Bañón *et al.* 2003).

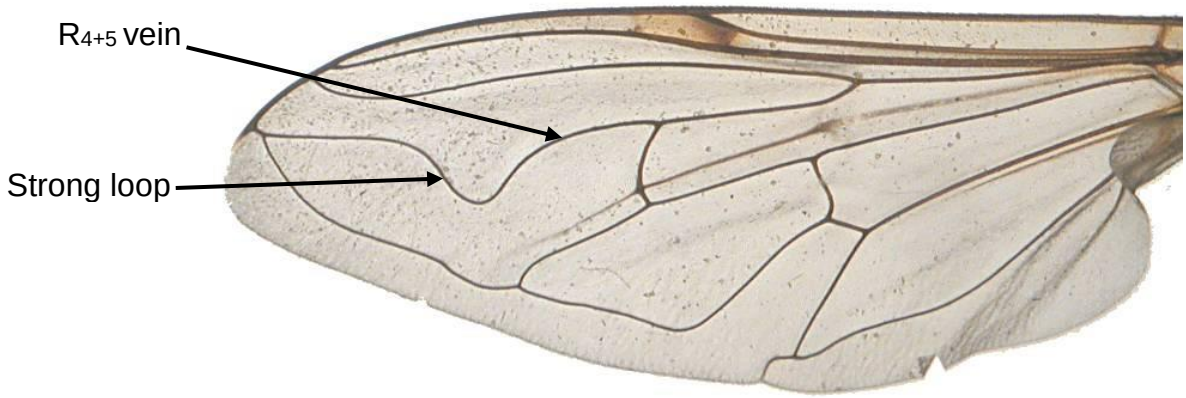


Figure 4. Here the strong loop of the R_{4+5} vein is shown, a character distinctive of the Eristalini tribe. © Roger Morris and Stuart Ball.

There has been previous work using molecular data when looking at Syrphidae as a whole, or to investigate the phylogeny of select tribes or genera. Ståhls and Nyblom (2000) used the mitochondrial gene cytochrome *c* oxidase subunit 1 (*cox1*) to investigate the phylogeny of the genus *Cheilosia,* a particularly diverse group of hoverflies. They were able to support various hypotheses, including the monophyly of *Cheilosia* and the division of *Cheilosia* in to two subgenera. The *cox1* gene is a popular choice to use as a molecular marker as there are regions that are both well conserved but also those with a high amount of variation, and so useful when exploring evolutionary relationships

and taxonomy between closely and distantly related species or groups (Lunt *et al.* 1996; Ståhls, 2000). Ståhls *et al.* (2003) used two genes, the *cox1* gene and the 28s rRNA gene as well as adult morphological characteristics to investigate various disputed taxonomic positions of different tribes and genera. Skevington and Yeates (2000) explored the wider superfamily Syrphoidea (Syrphidae and Pipunculidae) using mitochondrial 12s ribosomal DNA and 16s rDNA which resulted in the relationships within Syrphidae to be poorly supported, contrary to Pipunculidae where there was a good resolution of phylogenetic relationships.

Eristalis cryptarum is one of nine species in the *Eristalis* genus (*Eoseristalis* subgenus) and a morphologically distinct species, easily distinguished from other *Eristalis* species by the complete orange-red legs. Additional characteristics include its general smaller size and dark thorax and abdomen, the latter sporting yellow bands across the bottom of each tergite and orange hairs. Possessing an aquatic larval stage is one of the key characteristics of *Eristalis* species but as mentioned previously, the larvae of this species has yet to be discovered in the UK. To add to this gap in our knowledge of *E. cryptarum,* the use of molecular data to explore the phylogeny of this species has not yet been completed, in fact there has been no discussion of the phylogeny of *E. cryptarum* at all (to the authors knowledge).

1.3 Environmental DNA

Environmental DNA (eDNA) is a term that describes ‘free’ DNA that can be extracted from environmental samples without the need to locate and obtain a DNA sample from the actual organism in question (Taberlet *et al.* 2012). Environmental DNA (eDNA) is a relatively new definition that was first

introduced by the microbiology community in 1987 (Ogram *et al.* 1987) where microbial DNA was extracted and isolated from different types of sediment. In 1998, analysis of 'macrobial' eDNA first occurred when genetically modified tobacco plants were used to investigate plant DNA detection in soil samples (Paget *et al.* 1998) and eDNA analysis was then applied to fresh water for the first time where human, cow, pig and sheep DNA was detected in river water (Martellini *et al.* 2005).

Environmental samples can be collected from a variety of sources including: fresh water (Ficetola *et al.* 2008), seawater (Thomsen *et al.* 2012), soil (Yoccoz *et al.* 2012; Fahner *et al.* 2016), ice cores (Willerslev *et al.* 2007), air (Williams *et al.* 2001), faeces (Zhu *et al.* 2011; Guillerault *et al.* 2017), urine (Valiere & Taberlet, 2000), snow (Larose *et al.* 2010), blood meal (Schnell *et al.* 2012), honey (Schnell *et al.* 2010), cave sediments (Willerslev *et al.* 2003) and permafrost (Willerslev *et al.* 2003; Haile *et al.* 2009). One example of an environmental sample was demonstrated by Xu *et al* (2015) where eDNA was successfully amplified from spider webs. For both predator (black widow spiders *Latrodectus spp.*) and prey (house crickets *Acheta domesticus*), mitochondrial eDNA was extracted and amplified from spider webs and they showed that both spider and cricket DNA was amplifiable from spider webs 88 days after both were removed.

'Free' DNA is released by organisms in to the environment through several different ways, such as through: saliva, urine, faeces, blood, pollen, leaves, roots, root, fruit, decaying matter, skin, mucous, sperm, eggs, and other secretions (Bohmann *et al.* 2014). Any given environmental sample will contain the DNA of any number of different organisms and this will be mixture of both

cellular DNA and extracellular DNA released due to cell death and subsequent cell lysis (Levy-Booth, 2007; Bohmann *et al.* 2014).

The applications of eDNA

Utilising eDNA has been applied to a variety of studies. The use of eDNA from faecal matter and stomach contents can be used as an alternative to the use of stable isotopes and other approaches used in diet analysis (King *et al.* 2008; Yoccoz, 2012). Guillerault *et al.* (2017) collected the faeces and stomach contents of the European catfish *Silurus glanis* and used a DNA metabarcoding approach to identify the prey species composition in faecal samples. In total 14 different prey species were identified, 11 of which were identified by DNA metabarcoding. Understanding feeding habits is extremely useful in order to understand trophic interactions (Yoccoz, 2012; Guillerault *et al.* 2017).

The use of eDNA analysis has also been applied to biodiversity assessment studies. For example, Calvignac-Spencer *et al.* (2013) extracted and screened for mammalian eDNA from carrion feeding flies in order to explore this as a possibility for a large-scale mammalian monitoring method. They successfully amplified and detected 26 different species from a sample of 201 carrion flies. Leeches were used in a similar way to monitor mammalian biodiversity where data is deficient for many species that occur in the Central Annamite region of Vietnam (Schnell *et al.* 2012). Mammalian mitochondrial DNA from six different species was extracted and amplified from 21 out of 25 leeches collected, two of these species being newly described. This is an exciting example of an eDNA tool used for a biodiversity assessment study and highlights how quick and cost

effective the use of eDNA to survey biodiversity can be (Ficetola *et al.* 2008; Schnell *et al.* 2012; Port *et al.* 2016).

A popular and extremely beneficial application of eDNA is to detect the presence of invasive species. Goldberg *et al.* (2013) uses an eDNA methodology for the early detection of New Zealand mudsnails *Potamopyrgus antipodarum,* a highly invasive species in river systems which can reach high densities that subsequently harm ecosystem functions. The use of an eDNA methodology was also shown to be highly effective in the detection of the invasive American bullfrog *Lithobates catesbeianus* especially in comparison to traditional field sampling methods (Ficetola *et al.* 2008; Dejean *et al.* 2012). Piaggio *et al.* (2014) successfully managed to amplify Burmese python eDNA from water samples collected in Florida, where this species is invasive but also difficult to monitor using traditional field survey techniques due to its elusive nature. Invasive species have catastrophic effects of native biodiversity and can be incredibly expensive to eradicate (Dejean *et al.* 2012). Early detection of invasive species can therefore hugely beneficial and can make eradication more successful (Dejean *et al.* 2012).

As mentioned previously, environmental samples can include cave sediments, permafrost and ice cores. eDNA extracted from these types of environmental samples aid with the reconstruction of past flora and fauna (Bohmann *et al.* 2014), an alternative application of eDNA. In five permafrost cores collected from Siberia, the DNA of 19 different plant taxa as well as the DNA of mammoth, bison and horse was detected (Willerslev *et al.* 2003). DNA of 29 plant taxa and two species of extinct ratite moa was also successfully extracted and amplified from cave sediments collected in New Zealand (Willerslev *et al.* 2003). Willerslev *et al.* (2007) again was able to isolate DNA preserved in ice

cores collected from southern Greenland. Having extracted and identified the DNA of many conifer tree and insect species, they were able to reconstruct a previously theorised forest southern Greenland.

A further example of the application of eDNA techniques is for the detection of wildlife diseases. Chytridiomycosis *Batrachochytrium dendrobatidis* is one of the main explanations behind amphibian population declines and may survive in the environment without its host. Walker *et al.* (2007) developed an eDNA surveillance technique using filtration and qPCR methods and detected *B. dendrobatidis* DNA within and outside the distribution of the host species. As previous work relies on the surveying of infected hosts, the use of eDNA techniques allows the detection of this disease in the environment and therefore helps understand the lifecycle of this disease and the extinction risk for the host (Walker *et al.* 2007). Even though in this example whole organisms are being collected, as *Batrachochytrium dendrobatidis* DNA is being isolated from environmental sample, this is still an example of an eDNA technique. As mentioned previously, the first use of the term environmental DNA was applied to microbial DNA detection from environmental samples (Ogram *et al.* 1987) and so similar to this application. However, the term environmental DNA is still relevant to ‘free’ DNA released into the environment by organisms as addressed above.

The conservation implications of using eDNA as a survey tool

The key to the successful conservation is a thorough understanding of species distribution and ecology (Rees *et al.* 2014). The widely varied applications of eDNA analysis described above demonstrates the huge potential of eDNA and

the ability to detect early non-native species invasions (for example see: Ficetola *et al.* 2008; Goldberg *et al.* 2013; Piaggio *et al.* 2014), wildlife diseases (Walker *et al.* 2007) and rare or elusive species (e.g. Olsen *et al.* 2003; Thomsen *et al.* 2012; Piggott, 2017) without isolating the actual targeted organism via eDNA techniques has huge implications for conservation. Firstly, the use of eDNA can be more sensitive when compared to traditional methods (Dejean *et al.* 2012; Rees *et al.* 2014; Smart *et al.* 2015). For instance, Takahara, Minamoto and Doi (2013) used eDNA to detect the presence of the invasive bluegill sunfish *Lepomis macrochirus* and found eDNA was detected in all ponds where the species was observed and in ponds where *L. macrochirus* was not seen, indicating that eDNA can be more sensitive than traditional surveying techniques for the detection of species. Also, without the need to catch and isolate the target species, the use of eDNA as a survey tool is less invasive for the organism, a positive aspect in regards to the animal's welfare (Rees *et al.* 2014). It has been demonstrated that the use of eDNA as a survey tool can save considerable time and human effort. Jerde *et al.* (2011) used eDNA as a detection tool for two invasive species of Asian carp (*Hypophthalmichthys molitrix* and *H. nobilis*) and demonstrated that it took 0.174-person days to collect and process an eDNA sample, compared to 93 days it took to catch a single *H. molitrix* using electrofishing. Due to the sensitivity of eDNA analysis at lower population densities and therefore lower sampling efforts, this method is accepted to be a potentially cost-effect surveying method (Jerde *et al.* 2011; Rees *et al.* 2014; Wilcox *et al.* 2016).

As previously explained, for species that are particularly rare, endangered or elusive, the use of eDNA as a surveillance tool has become increasingly popular, due to the fact the species in question does not require isolation to be

genetically sampled. Olsen *et al.* (2012) investigated how effective eDNA sampling could be to monitor the eastern hellbender *Crypobranchus a. alleganiensis*, a species of conservation concern in eastern United States. Water samples were collected from streams where the hellbender is known to occur (shown by sampling in previous years) and filtered. Subsequent DNA extraction was performed on the filters used and primers developed in this study specific to this species were used in a series of PCR reactions. Eastern hellbender eDNA was successfully amplified in samples collected from sites where densities of eastern hellbender varied. Piggott (2017) used the eDNA approach in order to detect the presence of the endangered species, Macquarie perch *Macquaria australascia* in the Murray-Darling Basin, Australia. Primers had been developed for this species previously, but with some amplification in non-target species. Piggott (2017) used the mitochondrial cytochrome *b* gene to design primers specific to *M. australascia* that did not amplify DNA from co-distributed species. Primer specificity is extremely important as this limits the amplification of non-target species, therefore reducing the possibility of obtaining a false positive (Wilcox *et al.* 2013). Studies that use eDNA to survey species of conservation concern tend to be limited to vertebrate species (Roussel *et al.* 2015) but Thomsen *et al.* (2012) targeted 6 different locally rare aquatic species that occur in low abundance. These include amphibians: the common spadefoot toad *Pelobates fuscus* and great crested newt *Triturus cristatus,* fish: European weather loach *Misgurnus fossilis,* mammals: Eurasian otter *Lutra lutra,* insects: large white-faced darter *Leucorrhinia pectoralis* and a crustacean species: tadpole shrimp *Lepidurus apus.* The success rate of DNA detection for each species varied; the dragonfly species *L. pectoralis* had the lowest detection rate at 82%. The variability in detection rates between different

species is an important factor that should be considered while using eDNA methods (Roussel *et al.* 2015) but this study has demonstrated how eDNA techniques can be applied to a number of different taxa.

The drawbacks of using eDNA

Once DNA is released from an organism, it becomes very susceptible to environmental processes and therefore prone to environmental degradation (Strickler 2015). Environmental degradation, such as exposure to UV radiation, fluctuating temperatures and bacterial or fungal actions results in varying DNA degradation rates and nucleotide fragment lengths in environmental samples (Strickler *et al.* 2015). This will affect the detectability of eDNA and this detectability will vary depending on the environmental sample (Barnes *et al.* 2014). Strickler *et al.* (2015) kept bullfrog tadpoles (*Lithobates catesbeianus*) in different microcosms, and once the tadpoles were removed, exposed different microcosms to three different levels of temperature, ultraviolet B (UV-B) radiation and pH. Degradation of eDNA was evident in the first three to ten days but small amounts were still detectable after 58 days. Microcosms that were exposed to lower temperatures, lower UV-B radiation and more alkaline conditions had lower rates of eDNA degradation and therefore eDNA was detectable for longer. This study demonstrates how important environmental conditions are on the degradation rates of eDNA and adds to the complication of using an eDNA sampling technique for surveying. Pilliod *et al.* (2014) also look at the degradation rate of eDNA but also investigated the production and persistence rate of eDNA in water using the Idaho giant salamander *Dicamptodon aterrimus.* eDNA production rates were calculated by placing one

salamander per one aquarium (with known quantities of water) and water samples were collected from each aquarium per hour for the next five hours. eDNA production rates seemed to increase in the first 2 hours of the experiment (77ng per hour) which then slowed thereafter. The authors discussed a relationship between animal size and eDNA production (Thomsen *et al.* 2012), as well as factors such as stress causing the initial increase in eDNA production. Aquariums were exposed to both sunny and shaded conditions and eDNA was undetectable after three days where the samples were exposed to the sun, and eDNA was only detectable in 20% of samples collected from the shade. eDNA was able to be detected in all refrigerated control samples. Again, this is a clear example of how sensitive the detection of eDNA can be and this is an important factor which should always be taken in to account when using an eDNA approach to sampling.

Utilising eDNA in order to survey a species does not require isolating the organism in question, a major advantage and a particularly attractive aspect of using eDNA. However, it is possible to obtain a positive signal from an environmental sample where the species is actually present (Ficetola *et al.* 2016). False positives can occur due to a number of reasons, such as contamination while sampling, contamination in the laboratory and PCR or sequencing errors (Ficetola *et al.* 2015; Ficetola *et al.* 2016). In regard to surveying, this is an additional complication which traditional survey methods do not have (Roussel *et al.* 2015). Many protocols are put in place to eliminate the risk of contamination, therefore ensuring the elimination of false positives. This could include cleaning or replacement of equipment between sampling (Takahara, 2012), completing laboratory steps such as PCR in a room separated from other sources of DNA (Port *et al.* 2016) and importantly, having

controls at the point of sample collection, filtration and PCR amplification (e.g. Ficetola *et al.* 2008; Thomsen *et al.* 2012; Port *et al.* 2016).

Roussel *et al.* (2015) analysed literature using eDNA techniques and discusses the issues around eDNA studies. Other than the problem surrounding false positives as mentioned above, Roussel *et al.* (2015) highlights how 46% of the literature is focussed on fish species compared to only 8% of eDNA studies being focused on arthropods and so it is still uncertain whether or not the use of eDNA will be an effective tool for all aquatic species. Even more so, the detectability of different species isn't always addressed in each eDNA study, but it has been shown that some species cannot be detected solely using eDNA techniques when the species abundance is low (Tréguier *et al.* 2014). This can be problematic for species that are rare or exists in relatively low abundance.

1.4 Research aims and objectives

Eristalis cryptarum is a species of conservation concern. The key to the successful conservation of a critically endangered species is a thorough understanding and knowledge of the species behaviour, ecology and requirements (Rees *et al.* 2014). In this thesis, attempts will be made using molecular data to contribute to the knowledge gap surrounding *E. cryptarum.* **Chapter 2** will address the phylogeny of *E. cryptarum* with the use of mitochondrial molecular data and describe its taxonomic position in relation to other British hoverfly species collected throughout this project. In the absence of *E. cryptarum* larvae, exploring the taxonomic position of *E. cryptarum* will provide further support as to whether *E. cryptarum* larvae are aquatic or not. **Chapter 3** is focused on the design and development of species-specific

primers for *E. cryptarum* as well as other primer sets to amplify other hoverfly species in preparation for Chapter 3. With the knowledge *E. cryptarum* possesses an aquatic, rat-tailed larval stage, **Chapter 4** will utilise these species-specific primers to develop an environmental DNA methodology with the aim to screen for *E. cryptarum* specific eDNA in water samples collected from habitat sites on Dartmoor. The development of an eDNA methodology as a survey tool for *E. cryptarum* is a surveying method not yet attempted for this particularly rare and elusive species.

Chapter 2

The phylogeny of *Eristalis cryptarum* using mitochondrial cytochrome *c* oxidase subunit 1 gene (*cox1*) sequence data.

Abstract:

Eristalis cryptarum (Syrphidae, Diptera) is a UK Biodiversity Action Plan priority species and is critically endangered. The larvae of this species have yet to be discovered in the UK, and so the tailoring of conservation efforts to suitable habitats is difficult. Currently, on the basis of morphological characters, *E. cryptarum* has been taxonomically placed in the Eristalini tribe. If correct, then the assumption is that *E. cryptarum* larvae are aquatic, as this larval form is a distinguishing trait of the Eristalini tribe; such a deduction has significant implications for conservation. To test this assumption, we use the mitochondrial gene cytochrome *c* oxidase subunit 1 (*cox1*) to explore the phylogeny of *E. cryptarum* and confirm its placement within the Syrphidae family. *Agathomyia unicolor* (Platypezidae, Diptera) and *Nephrocerus flavicornis* (Pipunculidae, Diptera) were used as outgroups. We demonstrate strong support for *E. cryptarum* belonging in the Eristalini tribe and all species placed in the Eristalini tribe matched known phylogenies of this group based on morphology. This provides further support for the assumption that the larvae of *E. cryptarum* are aquatic and so the development of a robust phylogeny for *E. cryptarum* was an essential prerequisite for the development of an eDNA methodology utilising aquatic environmental samples to survey for *E. cryptarum* in Chapter 4.

Introduction:

Eristalis cryptarum (Syrphidae, Diptera) (Fabricius, 1784), commonly known as the bog hoverfly, is critically endangered and listed as a priority species in the UK Biodiversity Action Plan (Ball & Morris, 2014, Shirt, 1987, UK Biodiversity Group, 1999). In the UK, this species is now restricted to a number of sites across Dartmoor National Park in southwest England. The genus (*Eristalis*) to which this species belongs to is one of 8 genera in the Eristalini tribe (Subfamily Eristalinae): *Myathropa*, *Mallota*, *Helophilus*, *Eristalinus*, *Parhelophilus*, *Lejops*, *Anasimyia* and *Eristalis*. The Eristalini tribe is one of the most recognisable Syrphidae tribes with a pronounced loop easily visible on the R_{4+5} vein of the wing, a character only otherwise present in *Merodon* and a few other *Syrphus* groups (Fig.4). However, a further distinguishing morphological character of the Eristalini tribe is their aquatic larval stage. The larvae are known as 'rat-tailed' maggots, so named because of the long tail-like extension that is used as a breathing apparatus to allow the larvae to live in a variety of aquatic habitats. There has only been one reference (to the author's knowledge) of the larvae of *E. cryptarum* where pictures are shown (Kuznetsov, 1992), but this is a non-UK specimen and the larvae have yet to be discovered in the UK. The *Eristalis* genus is made up of two subgenera: *Eoseristalis* and *Eristalis* (Kanervo, 1938, Stubbs & Falk, 2002). Morphologically, the *Eoseristalis* subgenus is defined by the basal half of the hind tibia being pale and they lack noticeable hair fringes on the hind tibia (compared to *Eristalis tenax,* the only species found in the *Eristalis* subgenus). *Eristalis cryptarum* is one of nine species in subgenus *Eoseristalis* and is distinguished morphologically as a species by its small size, complete red-orange legs and orange hairs on its thorax, as well as a specificity to boggy and wet habitats on Rhôs pastures (Stubbs & Falk, 2002).

As the larval stage of *E. cryptarum* has not been described in the UK, it is important to determine the phylogeny of this species; if *E. cryptarum* is placed unequivocally in the Eristalini tribe then the assumption that the larval stage of this species is aquatic, as previously assumed (Drake & Baldock, 2005, Kuznetsov, 1992, Stubbs & Falk, 2002), is further supported. This an essential prerequisite for the development of an eDNA methodology to survey *E. cryptarum* eDNA in water samples, as larval eDNA will be targeted for screening in Chapter 4. This also has important implications for the conservation of this endangered species. A thorough understanding of the life cycle of a species is vital (New, 2007) and determining the form of the larval stage of *E. cryptarum* will aid in the understanding of larval requirements and breeding habitats.

The use of molecular markers in phylogenetic studies can offer advantages over the use of solely phenotypic characters. Firstly, obtaining molecular data for a specimen can sometimes be easier than obtaining the actual specimen itself, more so with accessible online databases such as GenBank and the Barcode of Life Database (BOLD) (Ratnasingham & Herbert, 2007). This is especially relevant when species are rare or threatened and obtaining a specimen for analysis is often impractical (Patwardhan, Ray & Roy, 2014). The molecular approach to phylogeny can also help resolve the relationships between species that are morphologically similar (Liu *et al.* 2018) or where there is phenotypic variation due to environmental factors that is not reflected at a molecular level, where such environmental influences are not expressed (Hillis, 1987).

The aim of this study was to investigate and confirm the phylogenetic placement of *E. cryptarum* using molecular data, which to the authors' knowledge has not

yet been undertaken for *E. cryptarum.* Here, the mitochondrial gene cytochrome *c* oxidase subunit 1 (*cox1*) was used. The *cox1* gene consists of a combination of highly variable and conserved regions while being one of three of the largest mitochondrial genes, making the *cox1* gene a suitable choice for exploring the evolutionary relationships between closely related taxa (Lunt *et al.* 1996). This gene is widely used as a DNA barcode for taxon and species identification (Herbert *et al.* 2003) and has been sequenced here for the first time for a UK specimen of *E. cryptarum*. The construction of a robust phylogeny for *E. cryptarum* and related taxa was an essential prerequisite for the development of species-specific primer sets necessary for the development of a reliable eDNA methodology (Chapter 3 and Chapter 4).

Materials & Methods:

Collection of specimens:

In total, 101 adult hoverfly specimens representing 31 different species were collected from Dartmoor National Park and Exeter (Devon, Southwest England) (Appendix, Table 8) during the period between September 2016 and September 2017. Hoverflies were caught using a sweep net and immediately placed in 100% ethanol at 4˚C prior to DNA extraction and amplification. Identification was performed by the author (unless otherwise states, see Table 3) prior to DNA extraction at the University of Exeter using identification guides (Ball & Morris 2015, Stubbs & Falk 2002).

DNA analysis:

Muscle fibres from the thorax of all hoverfly specimens were extracted and left to dry to allow full evaporation of any ethanol present. DNA was extracted from this tissue using QIAGEN DNeasy® Blood and Tissue Kit (Qiagen GmbH, Germany) following manufacturer's instructions. The DNA concentration and purity of the resultant extracts were quantified using a NanoDrop One (Thermo Scientific, Wilmington, DE, USA).

A fragment of the mitochondrial gene cytochrome oxidase *c* subunit 1 (*cox1*) was amplified using the following primers designed by Folmer *et al.* (1994): LCO1490 5'-GGTCAACAAATCATAAAGATATTGG-3' and HCO2198 5'-TAAACTTCAGGGTGACCAAAAAATCA-3'; the resultant fragment has a length of 658bp. *cox1cox1*PCR reactions were performed in 25 µl volumes with: 0.375 µl of each primer, 0.25 µl BSA, 12.5 µl HotStart Taq Master Mix (Qiagen), and 9 µl of RNase free water with 1 µl DNA extract. PCR was performed as follows: an initial denaturing temperature of 94˚C for 5 minutes, 35 cycles of 30 seconds denaturing at 94˚C, 30 seconds annealing at 50˚C and a 1-minute extension at 72˚C followed by a final extension for 7 minutes at 72˚C. A 5 µl aliquot of each amplified PCR product was separated using gel electrophoresis on a 1% agarose gel in order to check the success or failure of individual reactions. The remaining 20 µl of PCR product was also separated using gel electrophoresis on a 1% agarose gel and the target bands were isolated, extracted and purified using QIAquick® Gel Extraction Kit (Qiagen GmbH, Germany) following the manufacturer's instructions. The DNA concentration and purity of the resulting products was measured using NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Sequencing was completed externally by commercial sequencing facilities EUROFINS® (Eurofins Genomics, United

Kingdom) and GENEWIZ®. Additional sequences of further hoverfly species were obtained from GenBank and BOLD (Ratnasingham & Herbert, 2007).

Individual forward and reverse sequences for each specimen were examined by eye in Geneious 6.1.8 where sequences were edited and assessed for quality. After assessment, some sequences were trimmed and so shorter than 658 bp due to the quality of the sequence obtained (but see Table 3 for details on sequence lengths for each species analysed). Consensus sequences were produced using a pairwise alignment in Geneious which were then used to check sequences available on GenBank and BOLD to certify identification of the resultant consensus sequence. Two outgroup species were chosen: *Nephrocerus flavicornis (*Pipunculidae, Diptera), a member of the sister family to the hoverflies (Syrphidae, Diptera) and *Agathomyia unicolour* (Diptera, Platypezidae) which falls outside of the superfamily Syrphoidea (Syrphidae + Pipunculidae). Both species are found in the UK, and species from Pipunculidae and *A. unicolor* have been used as outgroups in previous studies exploring Syrphidae taxonomy and phylogeny (Rotheray & Gilbert 1998, Skevington & Yeates 2000, Ståhls *et al.* 2003). A multiple sequence alignment was performed in Geneious using CLUSTALW including the outgroup species. Where sequences were identical between different specimens of the same species, only one sequence was used in phylogenetic analysis. As well as this, sequences of poor quality were removed completely from analysis. And so not all specimens that were collected and underwent DNA extraction are used in phylogenetic analysis. See Table 3 for full information regarding the specimens used for subsequent phylogenetic analysis. *Phylogenetic analysis:*

Appropriate nucleotide substitution models were selected using MEGA 7.0.26 (Kumar, Stecher & Tamura 2015). GTR+G models were selected for this *cox1* dataset and phylogenetic analysis was performed in Geneious 6.1.8. Maximum Likelihood trees were constructed using the PhyML plugin (Guindon & Gascuel, 2003) in Geneious with the parameters set as follows: 100 bootstrap replicates, estimated proportion of invariable sites, gamma distribution parameter fixed at 0.23, an estimated transition/transversion ratio and the number of substitution rate categories set at 4. Bayesian phylogenetic trees were constructed using the MrBayes plugin in Geneious. The parameters were set as follows: a GTR substitution model was selected with gamma rate variation and 5 gamma categories. Four Monte-Carlo chains at a chain length of 1,100,000 with sampling frequency set at 200. Burn-in length was set at 100,000 and the selected outgroup was *Agathomyia unicolor.* The Consensus Tree Builder in Geneious was used to build consensus trees for both Maximum Likelihood and Bayesian reconstructions after removing the initial 10% burn-in and the support threshold set at 50%. Two morphological traits that are well known and accepted as distinguishing characters of the Eristalini tribe were selected and mapped on to both the Maximum Likelihood and Bayesian trees. The first morphological character used the presence of a strong loop on the R_{4+5} vein on the wing of the adult hoverflies (verified by identification guides, keys and visual examination of collected specimens) (Fig. 4) (Stubbs & Falk, 2002). The second morphological character mapped on to both trees was the presence of an aquatic, rat-tailed larval form (again, verified by identification guides, keys and visual examination of collected specimens) (Rotheray, 1993; Rotheray & Gilbert, 1999; Stubbs & Falk, 2002).

Results:

Out of the 101 specimens collected, 97 specimens underwent DNA extraction of which all gave positive PCR bands. These 97 specimens were sequenced and after resultant sequences were assessed for quality, 41 sequences representing 24 species were used in subsequent phylogenetic analysis. This number is low as some sequences were of poor quality and could not be used, or where sequences belonging to the same species matched completely, only one representative sequence was used. A further 26 sequences representing 14 species were obtained from BOLD or GenBank and used in subsequent phylogenetic analysis. In total, 67 sequences belonging to 38 different species were obtained and used in subsequent phylogenetic analysis (full specimen details in Table 3). Both the Maximum Likelihood tree (Fig. 5) and the Bayesian probability tree (Fig. 6) reconstructed using *cox1* sequences showed high support for monophyly of the Eristalini tribe (Maximum likelihood: 84% bootstrap support, Bayesian: 1.00 posterior probability) and species placed within this tribe were as hypothesised (Stubbs & Falk, 2002, Campoy *et al.* 2017). Within the Eristalini tribe, the *Eristalis* genus was well supported in the Bayesian probability tree but less so in the Maximum Likelihood tree. (Maximum Likelihood: 56% bootstrap support, Bayesian: 0.99 posterior probability). *E. cryptarum* was positioned within the *Eristalis* genus in both trees with 100% bootstrap support and a posterior probability of 1.00. Both morphological traits selected for mapping onto the trees agreed with the entire Eristalini tribe. Versions of both the Maximum Likelihood and Bayesian probability tree with all used specimens listed can be found in the Appendix.

The Syrphidae family is estimated to have diverged around 100 million years ago (Wiegman *et al.* 2011); thus, in the current study the root node separating

Agathomyia unicolour and *Nephrocerus flavicornis* from the Syrphidae family on the Bayesian tree has an estimated age of 100 million years ago. The same age can be estimated for the node separating *Nephrocerus flavicornis* from the Syrphidae family on the Maximum Likelihood tree. The Syrphidae family is reported to have experienced a rapid radiation around 50 million years ago (Wiegman *et al.* 2011).

Discussion:

Comments on the Eristalis genus and Eristalini tribe

To confidently determine the taxonomic placement of *E. cryptarum,* genus *Eristalis* and tribe Eristalini need to be defined unequivocally. The *cox1* gene appears well suited for distinguishing the Eristalini tribe for both Maximum Likelihood and Bayesian analysis. Furthermore, the morphological traits mapped onto the trees accord with the entire Eristalini tribe. One of these characters, the presence of the loop in the R_{4+5} vein (Fig. 4), is also found in a few other species belonging to the Syrphini tribe (this is indicated on Fig. 5 and Fig. 6; specific species possessing this trait are not individually represented). However, a combination of other morphological characters prevents the placement of these Syrphini species in the Eristalini tribe. For instance, the larval stages of the species in the Syrphini tribe are predacious, feeding on soft-bodied hemipterans (Mengual, Ståhls & Rojo, 2008) but the larvae of the Eristalini tribe are aquatic (in the form of rat-tailed maggots), feeding on decaying organic matter (Mengual, Ståhls & Rojo, 2008, Rotheray & Gilbert, 1999). The second morphological trait mapped on to the phylogenetic trees was the presence of an aquatic larval stage, specifically rat-tailed maggot larval form. Again, this trait is typical of the Eristalini tribe, however, *Sericomyia*

species also possess a rat-tailed larval stage (as shown on Fig. 5 and Fig. 6) Nevertheless, other combinations of morphological traits – such as a lack of a loop on the R_{4+5} wing vein on the adults and differing larval characters including: non-retractile anterior spiracles and small prolegs in the larval stage (Rotheray, 1993) – prevent the placement of *Sericomyia* species within the Eristalini tribe. And so, using a combination of two specific morphological traits and a *cox1* dataset, we are able to confirm the robustness of the Eristalini tribe.

The use of the mitochondrial gene *cox1* can have its limitations within phylogenetic studies. As Brown *et al.* (1979) explains, for species that have diverged over five to ten million years ago, the use of mitochondrial DNA can become less useful as a tool in phylogenetic studies. This is due to the accumulation of substitutions per base pair in mitochondrial DNA and so the accuracy of determining divergence times greatly reduces over longer periods of time. The Syrphidae are thought to have diverged approximately 100 million years ago and experienced rapid radiation around 50 million years ago (Wiegman *et al.* 2011). This may explain why resolution between different tribes is low in both trees constructed here using solely the *cox1* gene for the Syrphidae family. Despite this, we have still been able to distinguish the Eristalini tribe with high support and, as previously explained, all the species placed within the Eristalini tribe are as previously hypothesised and the selected morphological traits also correspond with this tribe. As discussed above, while a rapid divergence has been reported as having occurred in the Syrphidae within the last 50 million years (Wiegman *et al.* 2011), the strength of phylogenetic signal apparent in our *cox1* gene sequence data suggests that the Eristalini tribe may have diverged much later.

In terms of the other species outside of the Eristalini tribe, there are some tribes that have also been placed together using the *cox1* gene such as: Syrphini (including the species: *Syrphus ribesii, S. vetripennis, Eupeodes luniger, E. latifasciatus, Episyrphus balteatus* and *Chrystoxum bicinctum*), Sericomyinii (*Sericomyia silentis* and *S. lappona*) and Bacchini (*Melanstoma mellinum* and *M. scalare*). However, other species included in this analysis have not been grouped together by tribe. For example, *Rhingia campestris, Ferdinandea cuprea* and *Portevinia maculata* belong in the tribe Cheilosiini. However, in the Maximum Likelihood tree these species are not grouped together at all (Fig. 5) and in the Bayesian tree only *F. cuprea* and *P.* maculata are placed together with a lower posterior probability of 0.66 (Fig. 6). Ståhls *et al.* (2003) used the *cox1* gene and the nuclear 28S rRNA, together with both adult and larval morphological data to investigate the taxonomy of the Syrphidae family. Regarding taxa common to both their analysis and this current study, their findings support those species that have also been grouped together in our analysis (for example, the Eristalini and Syrphini tribe). The potential drawbacks of using only mitochondrial sequence data for older taxa is highlighted here, especially for tribes that may have diverged much earlier. In addition, in the current study sampling of taxa from other tribes was limited compared to the sampling of the species belonging to tribe Eristalini, as the primary focus of our research was on *E. cryptarum.*

The placement of Eristalis cryptarum

Establishing the taxonomic position of *E. cryptarum* using molecular data accords with previous studies regarding the placement of *E. cryptarum* within the Eristalini tribe (Stubbs & Falk, 2002). We demonstrate strong support in both the Maximum Likelihood and Bayesian phylogenetic trees with the use of the *cox1* gene. We also know that *E. cryptarum* possesses a definitive strong loop on the R_{4+5} vein verified by examination of our own specimens. This loop is a key identification characteristic for the Eristalini tribe (Fig. 4) and offers further morphological support for the taxonomic placement of *E. cryptarum* within the Eristalini tribe. By demonstrating that *E. cryptarum* belongs within the Eristalini tribe, we provide –albeit by association– further support that *E. cryptarum* is likely to possess an aquatic, rat-tailed larval stage. Previous work focused on the adult stages of *E. cryptarum* has shed light on the habitat typical of *E. cryptarum* and we know that the adult *E. cryptarum* are found in association with boggy habitats on Rhôs pastures (Drake and Baldock, 2002; Drake and Baldock, 2003; Drake and Baldock, 2004, Castle & Falk, 2013). However, some adult hoverfly species can be found far from where the larvae of the same species tend to occur. For example, *Eristalis tenax* is known to fly away from larval habitats to reach suitable flowering plants (Stubbs & Falk, 2002) and some hoverflies such as *Episryphus balteatus* has been known to be migratory species (Hart, Bale & Fenlon, 1997). Thus, it would appear prudent not to rush to assumptions when the larval stage has yet to be verified, particularly when there are important conservation implications relating to its larval form and preferred larval habitat (especially given its endangered status).

The adult life stage of hoverflies are generally uniform. They mostly feed on nectar and pollen and are seen as important pollinators (Moquet *et al.* 2018, Rader *et al.* 2016). However, the larval stages of hoverflies are massively

diverse. For example, the larvae of the subfamily Syrphinae (including the tribes Bacchini, Paragini and Syrphini) are predacious, feeding on aphids and other soft bodied homopterans (Rotheray, 1993). Other larvae are phytophagous (e.g. *Portevina maculata* feeding on bulbs of wild garlic), mycophages (e.g. *Cheilosia scutellata* breed in *Boletus* fungi) or saprophages (e.g. *Rhingia campestris* in cow dung) (Stubbs & Falk, 2002). Due to this huge amount of variation in the larval forms, previous taxonomic work on the Syrphidae has utilised larval characteristics (Rotheray & Gilbert, 1999; Skevington & Yeates, 2000; Stahls *et al.* 2003), which have proven an important aspect of Syrphidae phylogeny. Rotheray and Gilbert (1999), exploring the phylogeny of Palearctic Syrphidae using 187 larval morphological characters, demonstrated that all larvae that were aquatic were consistently grouped together, including the whole of the Eristalini tribe.

This study marks the first time that the phylogeny of *E. cryptarum* has been explored using molecular means, and constructing phylogenies using only morphological traits is difficult when not all life stages of a species are available for character scoring (Patwardhan, Ray & Roy, 2014). The *cox1* gene has long been used in phylogenetic studies of insects with success, for example for: Syrphidae (Ståhls *et al.* 2003, Ståhls *et al.* 2009), Calliphoridae (blowflies) (Stevens, 2003), Hemiptera (true, soft-bodied bugs) (Cui *et al.* 2012) and Formica (ants) (Chen & Zhou, 2017). Using a molecular approach in this scenario has allowed a ‘gap’ to be filled in knowledge of the larval stage of *E. cryptarum.*

Implications for the conservation of Eristalis cryptarum

Understanding the larval requirements of an endangered insect species is vital in order to be able to tailor conservation efforts effectively. It is all too easy to focus on the adult forms of a species, but without suitable breeding and larval habitats, insect populations will obviously struggle to maintain population numbers (Flockhart *et al.* 2015). For example, *Blera fallax* is an endangered species of hoverfly known as the pine hoverfly and is restricted to the Scottish Highlands. Breeding sites were successfully identified and described. The larvae filter feed in rotting Scots pine *Pinus sylvestris* stumps. Cavities are formed by the tree being weakened by the heart rot fungus *Phaeolus schweinitzi* which then fill with rain water (Rotheray & MacGowan, 2000). With the use of this knowledge, artificial breeding habitats were successfully created by creating holes in tree stumps and filling the artificial cavities with sawdust (Rotheray, Goulson & Bussière, 2016).

We have provided strong molecular support for the placement of *E. cryptarum* in the Eristalini tribe. This contributes support to the assumption that the larva of *E. cryptarum* has a rat-tailed aquatic larval stage. Significant knowledge in regard to the habitat requirements and characteristics of the species can be gained through observations of adult *E. cryptarum.* Adult *E. cryptarum* are found along valley mires in Rhôs pastures as previously explained and have been observed feeding on a number of wildflowers also specific to this habitat, e.g. bog asphodel *Narthecium ossifragum* and marsh marigold *Caltha pulustris.* This combined knowledge about this critically endangered species will help tailor surveying efforts to these specific habitats and aid in the search for the larval stage of *E. cryptarum*, ultimately helping to target conservation efforts effectively at breeding sites crucial for the long-term protection of this enigmatic and beautiful species. In regard to this thesis, the construction of a robust

phylogeny for *E. cryptarum* and related taxa was essential in preparation for the development of an eDNA surveying tool targeting *E. cryptarum* in aquatic environmental samples (Chapter 4) as well as providing the data needed to develop species-specific primer sets (Chapter 3) necessary for eDNA analysis.

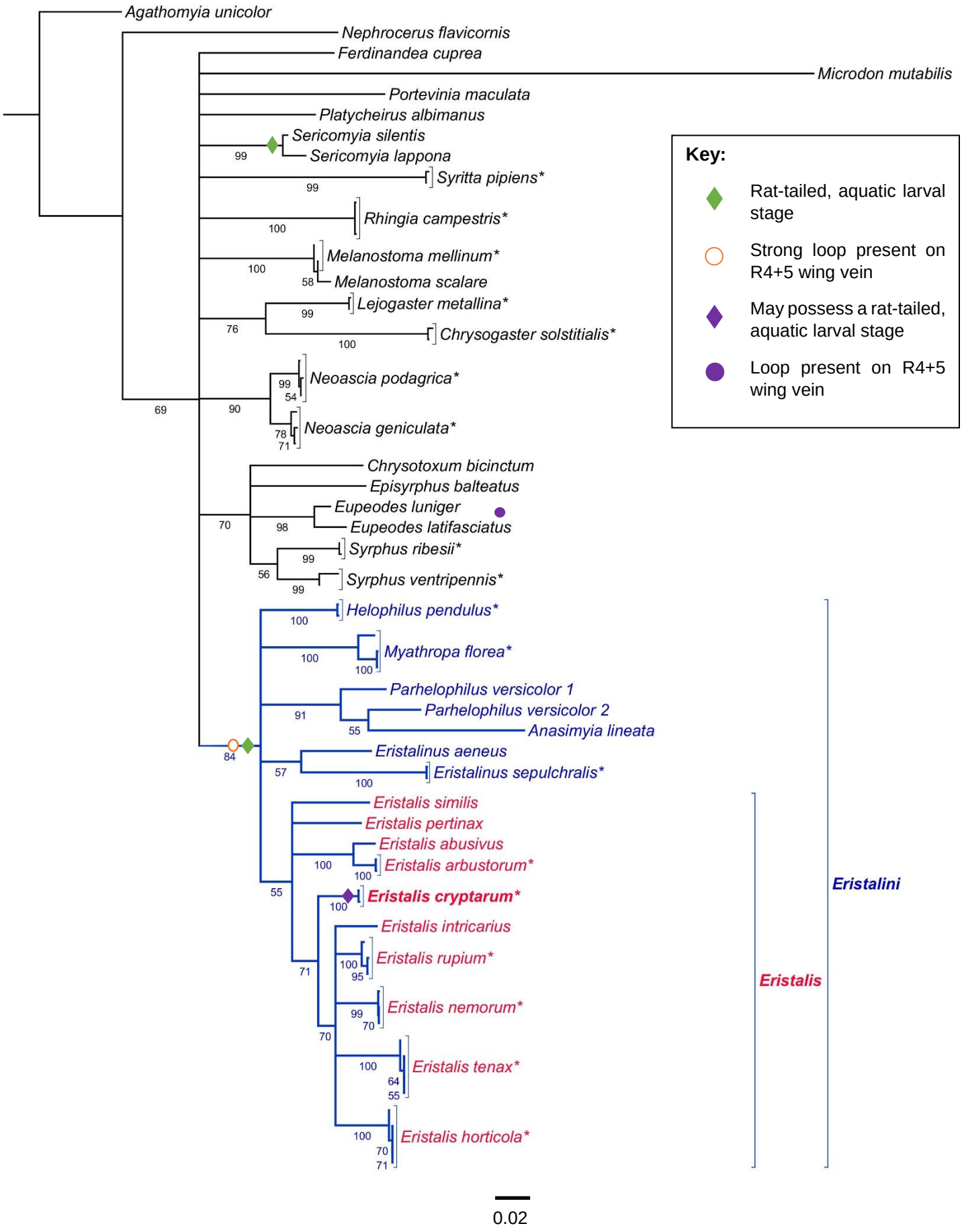

Agathomyia unicolor
Nephrocerus flavicornis
Ferdinandea cuprea
Microdon mutabilis
Portevinia maculata
Platycheirus albimanus
Sericomyia silentis
Sericomyia lappona
Syritta pipiens*
Rhingia campestris*
Melanostoma mellinum*
Melanostoma scalare
Lejogaster metallina*
Chrysogaster solstitialis*
Neoascia podagrica*
Neoascia geniculata*
Chrysotoxum bicinctum
Episyrphus balteatus
Eupeodes luniger
Eupeodes latifasciatus
Syrphus ribesii*
Syrphus ventripennis*
Helophilus pendulus*
Myathropa florea*
Parhelophilus versicolor 1
Parhelophilus versicolor 2
Anasimyia lineata
Eristalinus aeneus
Eristalinus sepulchralis*
Eristalis similis
Eristalis pertinax
Eristalis abusivus
Eristalis arbustorum*
Eristalis cryptarum*
Eristalis intricarius
Eristalis rupium*
Eristalis nemorum*
Eristalis tenax*
Eristalis horticola*
Eristalini
Eristalis
Key:
Rat-tailed, aquatic larval stage
Strong loop present on R4+5 wing vein
May possess a rat-tailed, aquatic larval stage
Loop present on R4+5 wing vein
0.02

Fig. 5 Maximum Likelihood tree constructed using a *cox1* dataset. 67 *cox1* sequences were used. Values below branches show bootstrap support values (%). Both the Eristalini tribe and *Eristalis* genus is highlighted. ○ indicates the presence of a strong loop on the R4+5 wing vein in adults and ● indicates that a loop exists on this vein, but not as evident (specific species possessing this trait are not individually represented, ● symbol is instead placed on the relevant tribe by the species that is most related to those species that do possess this trait). Species that possess an aquatic, rat-tailed larval stage are indicated by ♦ and if this is currently unknown, this is shown by ♦. * indicates that more than one sequence belonging to the same species was used and a Maximum Likelihood tree showing all sequences used can be found in the Appendix (Fig. 11). To see which specimens were used that were collected by (or donated to) the author, specimens are indicated in bold font on Table 3, Fig. 11 (Appendix) and Fig. 12 (Appendix).

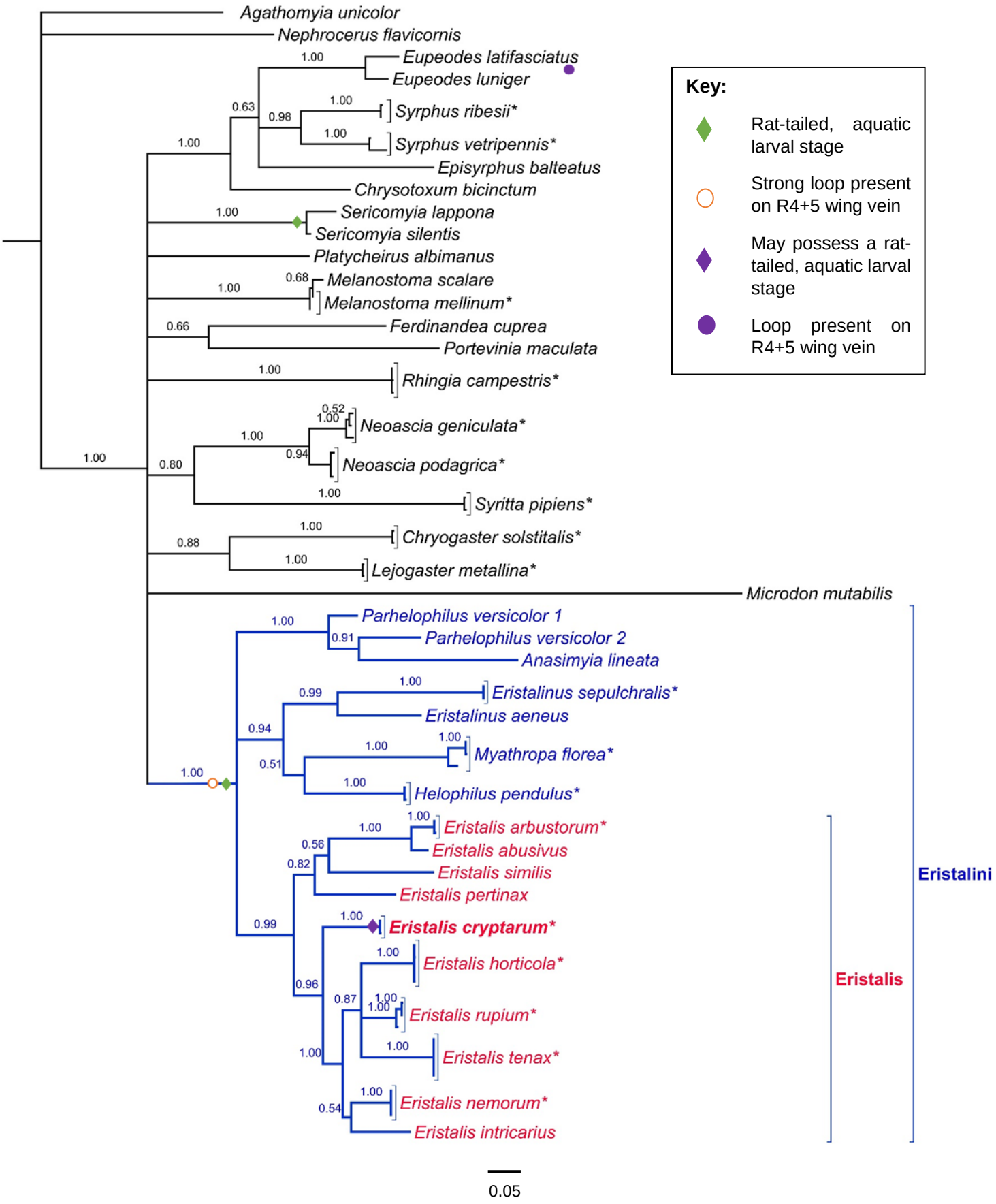


Fig. 6 Bayesian probability tree constructed using a *cox1* dataset. 67 *cox1* sequences were used. Values below branches show posterior probability

values. Both the Eristalini tribe and *Eristalis* genus is highlighted.◯ indicates the presence of a strong loop on the R4+5 wing vein in adults and ● indicates that a loop exists on this vein, but not as evident (specific species possessing this trait are not individually represented,● symbol is instead placed on the relevant tribe next to the species most closely related to the species that possess this trait). Species that possess an aquatic, rat-tailed larval stage are indicated by and if this is currently unknown, this is shown by ♦. ***** indicates that more than one sequence belonging to the same species was used and a Maximum Likelihood tree showing all sequences used can be found in the Appendix (Fig. 11). To see which specimens were used that were collected by (or donated to) the author, specimens are indicated in bold font on Table 3, Fig. 11 (Appendix) and Fig. 12 (Appendix).

Table 3 Details of all specimens used in phylogenetic analysis. Details for each specimen used include: collection location, GenBank accession number or BOLD ID where applicable and initials of those who identified each specimen. Specimens identified by Catherine Mitson (CM) or (JW) are specimens collected during this study and are in bold. For specimens collected throughout this project, more collection details are given in Table 8 where the specimens used in Chapter 1 are in blue.

Specimen	ID	Collection location	Accession no. /BOLD ID	Sequence length (bp)
Agathomyia unicolor	GS	Finland	LN623685.1	660
Nephrocerus flavicornis	CK	Bialowieska National Park, Poland	FM213137.1	658
Ansimyia lineata	**JW**	**Corndonford, Dartmoor, UK**		**658**

Table 3 continued

Specimen	ID	Collection location	Accession no. /BOLD ID	Sequence length (bp)
Chrysogaster solstitialis 1	FO	Monaryggen, Norway	NORSY474-15	658
Chrysogaster solstitialis 2	FO	Asaktoppen, Norway	NORSY475-15	658
Chrysotoxum bicinctum	**JW**	**Pizwell, Dartmoor, UK**		**658**
Episyrphus balteatus	**CM**	**Exeter, Devon, UK**		**658**
Eristalinus aeneus	**CM**	**Coleton Fishacre, Devon, UK**		**658**
Eristalinus sepulchralis 1	RO	Abergwyngregyn, UK	BEEEE373-16	604
Eristalinus sepulchralis 2	FO	Kongsgardmoen, Norway	NORSY397-12	658
Eristalis abusivus	JW	St Davids, Wales		606
***Eristalis arbustorum* 1**	**CM**	**Exeter, Devon, UK**		**657**
***Eristalis arbustorum* (larvae) 2**	**CM**	**South Devon, UK**		**624**
***Eristalis cryptarum* 1**	**CM**	**Corndonford, Dartmoor, UK**		**636**
Eristalis cryptarum 2	**CM**	**Corndonford, Dartmoor, UK**		**658**
***Eristalis horticola* 1**	**CM**	**Buckland Common, Dartmoor, UK**		**617**
***Eristalis horticola* 2**	**CM**	**Buckland Common, Dartmoor, UK**		**657**
***Eristalis horticola* 3**	**CM**	**Buckland Common, Dartmoor, UK**		**657**
***Eristalis horticola* 4**	**CM**	**Buckland Common, Dartmoor, UK**		**658**
Eristalis intricarius	**JW**	**Corndonford, Dartmoor, UK**		**631**
***Eristalis nemorum* 1**	**JW**	**Moortown, Dartmoor, UK**		**650**
***Eristalis nemorum* 2**	**JW**	**Moortown, Dartmoor, UK**		**652**
***Eristalis nemorum* 3**	**CM**	**Exeter, Devon, UK**		**657**
Eristalis pertinax	**JW**	**Corndonford, Dartmoor, UK**		**627**
Eristalis rupium 1		Riding Mountain National Park, Canada	TTDFW218-08	658

Table 3 continued

Specimen	ID	Collection location	Accession no. /BOLD ID	Sequence length (bp)
Eristalis rupium 2	FO	Stordalsberget, Norway	NORSY175-12	658
Eristalis rupium 3		Yoho National Park, Canada	BBDCP816-10	638
Eristalis similis	FO	Hamresanden, Norway	NORSY488-15	658
***Eristalis tenax* 1**	**CM**	**Pizwell, Dartmoor, UK**		**638**
***Eristalis tenax* 2**	**CM**	**Pizwell, Dartmoor, UK**		**658**
***Eristalis tenax* 3**	**CM**	**Pizwell, Dartmoor, UK**		**657**
***Eristalis tenax* 4**	**CM**	**Pizwell, Dartmoor, UK**		**655**
Eupeodes latifasciatus	**CM**	**Exeter, Devon, UK**		**658**
Eupeodes luniger	**CM**	**Exeter, Devon, UK**		**658**
Ferdinandea cuprea	**CM**	**Exwick, Devon, UK**		**658**
***Helophilus pendulus* 1**	**CM**	**Pizwell, Dartmoor, UK**		**658**
***Helophilus pendulus* 2**	**CM**	**Exeter, Devon, UK**		**652**
Lejogaster metallina 1	AV	England, UK	BEEEE381-16	612
Lejogaster metallina 2	FO	Jomfruland, Norway	NORSY092-12	658
***Melanostoma mellinum* 1**	**CM**	**Exeter, Devon, UK**		**658**
***Melanostoma mellinum* 2**	**CM**	**Exeter, Devon, UK**		**639**
Melanostoma scalare	**CM**	**Exeter, Devon, UK**		**658**
Microdon mutablis	**JW**	**Pizwell, Dartmoor, UK**		**658**
Myathropa florea 1	FO	Fredriksten Festning, Norway	NORSY109-12	658
Myathropa florea 2		Ilkley, UK	BEEEE383-16	612
Myathropa florea 3	FO	Jomfruland, Norway	NORSY066-12	658
Neoascia geniculata 1	JW	Ripley beach, Canada	JWDCG881-10	658
Neoascia geniculata 2	JS	Churchill, Canada	KR671770.1	658
Neoascia geniculata 3	FO	Faksfall, Norway	NORSY519-15	658

Table 3 continued

Specimen	ID	Collection location	Accession no. /BOLD ID	Sequence length (bp)
Neoascia podagrica 1	JV	Lifta, Israel	JN992017.1	658
Neoascia podagrica 2	AV	England, UK	BEEE398-16	612
Neoascia podagrica 3	FO	Jomfruland, Norway	NORSY083-12	658
Parhelophilus versicolor 1	FO	Hamresanden, Norway	NORSY447-15	658
Parhelophilus versicolor 2	CA	Selkirk, Scotland, UK	BEEE410-16	612
Platycheirus albimanus	**CM**	**Exeter, Devon, UK**		**658**
Portevinia maculata	**RW**	**Coleton Fishacre**		**658**
***Rhingia campestris* 1**	**CM**	**Pizwell, Dartmoor, UK**		**658**
***Rhingia campestris* 2**	**CM**	**Pizwell, Dartmoor, UK**		**633**
***Rhingia campestris* 3**	**CM**	**Buckland Common, Dartmoor, UK**		**658**
Sericomyia lappona	FO	Stordalsberget, Norway	NORSY177-12	658
Sericomyia silentis	**CM**	**Pizwell, Dartmoor, UK**		**658**
Syritta pipiens 1	FO	Busund, Norway	NORSY026-12	658
Syritta pipiens 2		Sheck Nature reserve, Canada	KR682638.1	658
***Syrphus ribesii* 1**	**CM**	**Exeter, Devon, UK**		**658**
***Syrphus ribesii* 2**	**CM**	**Exeter, Devon, UK**		**658**
***Syrphus vetripennis* 1**	**CM**	**Exeter, Devon, UK**		**658**
***Syrphus vetripennis* 2**	**CM**	**Exeter, Devon, UK**		**611**

Chapter 3

Development of species-specific primers for the detection of hoverfly environmental DNA

Abstract:

The molecular detection of a specific species using environmental DNA (eDNA) has proved to be successful in a number of different applications. Integral to this is the development of species-specific PCR primers, a vital aspect of eDNA studies. The specificity and sensitivity of the taxon-specific primers designed should be explored and established before being used in wider eDNA studies. Typically, in an environmental sample, the eDNA of many organisms will be found, and so the ability of primers to amplify the eDNA of the target species must be understood. Here, a species-specific primer set will be designed and developed for the bog hoverfly *Eristalis cryptarum,* an endangered hoverfly species which, in the UK, is found only in Dartmoor National Park, Devon. A taxon-specific primer set was also developed for the *Eristalis* genus, as well as a final species-specific primer set for a closely related species, *E. arbustorum.* The specificity of these primers to their target species or taxa were tested against different hoverfly taxa, followed by a number of dilution series to explore the sensitivity of each primer set. *E. cryptarum* DNA was also mixed with another *Eristalis* species, *E. horticola*, at varying ratios to test how sensitive the *E. cryptarum*-specific primers are at amplifying *E. cryptarum* against other closely related species. This was undertaken in preparation for research continued in Chapter 4, whereby *E. cryptarum* larval eDNA was screened for in

environmental water samples collected from presumed *E. cryptarum* habitat sites across Dartmoor.

Introduction:

Most, if not all, surveying techniques possess some degree of error. For example, during field surveys, misidentification of a species can lead to a species being recorded where it is in fact absent. This is a false positive error and can lead to an overestimation of a species distribution (McClintock *et al.* 2010). Alternatively, false negatives can occur, where detection of a species may not be recorded where it is actually present (McClintock *et al.* 2010). The detection of DNA from environmental samples has been applied successfully to a number of different scenarios as a species surveying tool. Species detection is arguably the biggest application of this technique, in order to monitor the distribution and presence of invasive, rare or elusive species (for example see: Ficetola *et al.* 2008; Olsen *et al.* 2012; Goldberg *et al.* 2013), but eDNA has also been applied to the detection of wildlife diseases (Walker *et al.* 2007), the reconstruction of past flora and fauna (Willerslev *et al.* 2003) and to explore the diets of organisms (Guillerault *et al.* 2017). When targeting a specific species or taxon in environmental DNA studies, the specificity of the primers used is extremely important. Only DNA of the target taxa should be amplified if present, as amplification of non-target species can lead to false positives and therefore provide an incorrect estimate of the distribution, presence or abundance of the target species (Darling & Mahon, 2011; Ficetola, Taberlet & Coissac, 2016). In addition, the sensitivity of the primers used in eDNA should also be explored, so that there is an understanding of how well primers perform with varying degrees of DNA concentrations of the target taxa present in environmental samples, as well as with particularly degraded eDNA (Roussel *et al.* 2015; Macdonald & Sarre, 2017).

In Chapter 3, an eDNA methodology is used to determine the presence or absence of *Eristalis cryptarum* eDNA in water samples. *E. cryptarum,* commonly known as the bog hoverfly, is a critically endangered species and a priority species on the UK Biodiversity Action Plan (UK Biodiversity Group, 1999). The larvae of this species are presumed to be aquatic, as explored in Chapter 2. Chapter 3 presents the development of a species-specific primer set for *E. cryptarum* that ultimately only amplifies larval *E. cryptarum* eDNA; this is in preparation for the application of a broader eDNA survey of *E. cryptarum* from environmental water samples in Chapter 4. In addition, a PCR primer set will be developed for the *Eristalis* genus (all the species of which also possess a larval stage) to use alongside *E. cryptarum* primers to aid in distinguishing potential false negative and false positive results. Additionally, a specific primer set for *E. arbustorum,* a closely related species to *E. cryptarum*, will be designed to facilitate unambiguous identification of these two species. A fragment of the mitochondrial gene cytochrome *c* oxidase subunit 1 (*cox1*) will be targeted. The *cox1* gene is widely used as a DNA barcoding and species identification tool (Herbert, Ratnasingham, deWaard 2008). Furthermore, the use of mitochondrial DNA (mtDNA) has added advantages in eDNA studies because per cell there are more copies of a target gene located in mtDNA compared to nuclear DNA. In addition, the mitochondrion itself provides added protection for mtDNA against environmental degradation once it is released in to the environment (Wilcox *et al.* 2013; Turner *et al.* 2014). Following primer development, the sensitivity of these primers was investigated in laboratory conditions through a dilution series. Once designed and validated, these primer sets will be used in Chapter 3 to develop an eDNA methodology to screen for *E.*

cryptarum eDNA in water samples collected from known habitat sites in Dartmoor National Park, Devon, UK.

Materials and Methods:

Collection of specimens

E. cryptarum has a flight period between May and early October (Drake & Baldock, 2005; Ramel, 1998; Perrett, 2001). During this period, frequent visits were made to known *E. cryptarum* habitat sites (Table 1). During these field visits, other species of hoverflies were caught using a sweep net and stored in 100% ethanol at 4˚C until subsequent DNA extraction (full details of collected specimens can be found in Table 8). *E. cryptarum* is an extremely elusive species and is reported as flying away quickly at the smallest disturbance (Drake, 2005). The most effective method was the 'sit and wait' approach and to use a sweep net to catch individuals sunning themselves on the sphagnum moss characteristic of this habitat. After two months of searching from the start of the adult flight period in May 2017, three male *E. cryptarum* specimens were collected in total throughout July 2017 and stored in the same manner described above. Due to the conservation status of this species, collecting more than three specimens was deemed unnecessary. In addition to the hoverfly specimens collected, *E. arbustorum* larvae were also collected. *E. arbustorum* is closely related to *E. cryptarum* and possess an aquatic, rat-tailed maggot larval stage. Larvae of this species were collected from Gara Rock beach, South Devon and brought back to the laboratory. Larvae were kept in a known volume of water of 50 ml and supplied with constant feeding material (decaying matter from where larvae were found and dead leaf material). Water used to

maintain larvae was collected from a clear running stream from Exeter University campus. Streams are not the habitat of *Eristalis* larvae (Stubbs & Falk, 2002) and so ensures the absence of *Eristalis* larvae in water used here. These larvae and the water they were maintained in were subsequently used in the development of the eDNA methodology presented in Chapter 3. All specimens were identified by the author in the laboratory using the identification guide British Hoverflies (Stubbs & Falk, 2002) or pre-identified specimens were donated to the author (Table 8).

Primer design:

DNA extractions of adult hoverfly specimens (Table 8) and subsequent PCR reactions and gel electrophoresis were performed as described in Chapter 2 using the primers designed by Folmer *et al.* (1994). Sequencing was completed externally by commercial sequencing facilities EUROFINS® (Eurofins Genomics, United Kingdom) and GENEWIZ®. Additional sequences of further hoverfly species were obtained from GenBank and BOLD (Ratnasingham & Herbert, 2007). Individual forward and reverse sequences of the *cox1* gene for each specimen were edited and assessed for quality in Geneious 6.1.8. Using a pairwise alignment, consensus sequences were produced and checked against available sequences in GenBank and BOLD for verification. A multiple species alignment was performed for all sequences in Geneious using CLUSTALW. Three primer sets were designed by eye using the multiple alignment produced within Geneious. Each primer set was tested against the multiple alignment using Primer3 in Geneious (Koressaar & Remm, 2007; Untergasser, 2012).

Primer specificity was further confirmed using Primer-BLAST (Ye *et al.* 2012). The designed primer sets for all target taxa are listed in Table 5.

1) *Eristalis* genus primer set

Firstly, a primer set was developed in order to amplify a fragment of the *cox1* gene of species belonging to the *Eristalis* genus. This is so that the presence of *E. cryptarum* can be verified by a double positive. If there is no *E. cryptarum* DNA present in the sample, the presence or absence of other hoverflies can be distinguished; this strategy was employed to determine potential false negatives as well as demonstrating the ability to amplify hoverfly eDNA. These primers, ErisF2 and ErisR1, were tested and an optimum annealing temperature of 50˚C was used in subsequent PCR reactions; a resultant 202 base pair (bp) portion of the *cox1* gene was amplified (Table 5). Firstly, this primer set was used with all available *Eristalis* genus specimens. Primer sets were then tested against other available hoverfly species that possess an aquatic larval stage. PCRs were performed in 10 µl volumes: 5 µl HotStart Taq Master Mix (Qiagen), 3.5 µl RNase free H_20, 0.2 µl of each primer, 0.1 µl BSA and 1 µl of the DNA template. PCR was performed as follows: an initial denaturing temperature of 94˚C for 5 minutes, 35 cycles of 30 seconds denaturing at 94˚C, 30 seconds annealing at 50˚C and a 1 minute extension at 72˚C followed by a final extension for 7 minutes at 72˚C. The sensitivity of the ErisF2 and ErisR1 primer set was also investigated by performing a dilution series using DNA from four specimens: Etenax1, Ecryptarum1, Ehorticola1 and Earbustorum1 (Table 6). Initial DNA concentrations were: 55.3 ng/µl, 17.8 ng/µl, 33.6 ng/µl and 93.3 ng/µl, respectively (Table 6). These were each diluted by a factor of ten up to a 10^{-6} dilution and PCR amplifications were performed as above. PCR products were

separated by electrophoresis on a 1% agarose gel to visualise the success or failure of individual reactions.

2) *E. arbustorum* primer set

An *E. arbustorum*-specific primer set was also designed. An annealing temperature of 55˚C was deemed optimal after testing and successful amplification of a 181 bp fragment of the *cox1* gene of *E. arbustorum.* This primer set was tested with other *Eristalis* species and specimens of other British aquatic hoverfly species. PCR reactions were performed in 10 µl volumes as above but with a 30 second annealing period at 55˚C. The sensitivity of the EarbusF1 and EarbusR1 primer set was also investigated by performing a dilution series using DNA from two specimens: Earbustorum1 and Earbustorum2 (Table 6). Initial DNA concentrations were: 89.3 ng/µl and 93.3 ng/µl. These were each diluted by a factor of ten up to a 10^{-6} dilution and, again, PCR amplifications were performed as above. PCR products were separated using gel electrophoresis on a 1% agarose gel to visualise success or failure of individual reactions.

3) *E. cryptarum* primer set

Finally, a primer set specific to *E. cryptarum* was designed. To avoid false positives, it was critical that this primer set only amplified *E. cryptarum*. The resultant fragment amplified with this species-specific primer set had a length of 170 bp. As above, these primers were tested against the *Eristalis* genus specimens and other hoverfly species with an aquatic larval stage to determine primer specificity. PCRs were performed in 10 µl volumes as above but with a 30 second annealing period at 57˚C. PCR products were separated by

electrophoresis using a 1% agarose gel to visualise the success or failure of individual reactions.

In addition to the primer specificity test performed above, the DNA of *E. cryptarum* was spiked with the DNA of a closely related species *E. horticola.* . An aliquot of *E. cryptarum* DNA extract (17.8 ng/µl) was mixed at varying ratios with *E. horticola* DNA extract (33.6 ng/µl) (Table 4). This species was selected due to the author's observations of female *E. horticola* patrolling potential egg-laying habitat where *E. cryptarum* may also be breeding and so, in the eDNA study presented in Chapter 3, *E. horticola* eDNA may also be present. Furthermore, *E. horticola* is a closely related species to *E. cryptarum* (see Chapter 3), so would provide additional demonstration of the specificity of this primer set. The specificity of these primers have still been tested against the *Eristalis* genus and other hoverfly species with an aquatic larval stage (see above). However, the spiking of *E. cryptarum* DNA with another species DNA further demonstrates the ability of these primers to amplify *E. cryptarum* DNA even if the DNA of another closely related hoverfly species is found in higher quantities than *E. cryptarum* in an environmental sample. Six PCR replicates were performed, and PCRs were performed in 10 µl volumes as above. However, for each PCR replicate, the 1 µl DNA extract used was made up of varying ratios of *E. horticola* and *E. cryptarum* DNA (Table 4). PCR was performed as above (with a 30 second annealing step at 57˚C). The sensitivity of the EcrypF3 and EcrpR4 primer set was also investigated by performing a dilution series using DNA from three specimens: Ecryptarum1, Ecryptarum2 and Ecryparum3 (Table 6). Initial DNA concentrations were: 28.5 ng/µl, 17.8 ng/µl, 65.8 ng/µl (Table 6). These were each diluted by a factor of ten up to a

10^{-6} dilution and PCR amplifications were performed as above. All PCR products were separated by electrophoresis on a 1% agarose gel to visualise the success or failure of individual reactions.

Table 4 Percentage of *E. cryptarum* and *E. horticola* DNA extract used in a 1 µl DNA aliquot for each of the six PCR replicates.

Species	Percentage of DNA used					
E. cryptarum	100	80	60	40	20	0
E. horticola	0	20	40	60	80	100

Table 5 The primer sets designed for each of the target taxa. The optimal annealing temperatures for PCR amplification is given as well as the resultant CO1 fragment length.

Target species	Primer name	Sequence 5-3’	Fragment (bp)
Eristalis sp.	ErisF2	GCTGAATTAGGHCATCCTGGA	202
	ErisR1	TTTTCTACTATACTTCTTACTA	
Eristalis arbustorum	EarbF1	GAACAGTTTATCCACCTT	181
	EarbR1	GAACTGGAAGTGATAAAAGAAGTAATAAA	
Eristalis cryptarum	EcrypF3	AGTTTACCCTCCTTTATCAAGTAAT	170
	EcrypR4	AAACTGGTAATGAAAGAAGAAGTAATAAA	

Results:

1) *Eristalis* genus primer set

The primer set ErisF2 and ErisR1 successfully amplified a portion of the *cox1* gene in all species of the *Eristalis* genus tested. When used with other hoverfly specimens that also possess an aquatic or semi-aquatic larval stage, two out of four species also amplified: *Rhingia campestris* and *Neoascia tenur.* Due to the limited amount of variation in the *cox1* gene a more specific primer for the *Eristalis* genus was unable to be designed. Increasing the annealing temperatures in the PCR protocol resulted in an increase in the specificity to the *Eristalis* genus, however, at the same time the effectiveness of the primers' ability to amplify the *Eristalis* genus started to decrease. In the dilution series, these primer sets were able to amplify reliably down to a 10^{-2} dilution, while still producing a visible, but faint reading at a dilution of 10^{-3} (Fig. 7).

2) *E. arbustorum*-specific primer set

EarbF1 and EarbR1 specificity was firstly determined through Primer-BLAST (Ye *et al.* 2012). The first nine results with no mismatches were a mix of *E. arbustorum* and general Diptera species but further investigation of these sequences confirmed them all to be *E. arbustorum.* The tenth result suggested the presence of *E. brousii*, but this is a non-UK species (Telford, 1970). At the annealing temperature of 55˚C, the EarbF1 and EarbR1 primer set successfully amplified *E. arbustorum* DNA. No other *Eristalis* species or hoverflies that possess an aquatic larval stage were amplified. In the dilution series, a very strong band was displayed down to a dilution of 10^{-3}. Subsequent to this, the

ability of these primers to amplify below this dilution decreased rapidly (only one faint band was present for one specimen at 10^{-4}) (Fig. 8).

3) *E. cryptarum*-specific primer set

EcrypF3 and EcrypR4 primer specificity was firstly confirmed using Primer-BLAST (Ye *et al.* 2012) where the first nine matched *E. cryptarum* with no mismatches and the tenth result was *E. fraterculus* with two mismatches (a non-UK species). *E. cryptarum* was successfully amplified at an annealing temperature of 57˚C. No species in the *Eristalis* genus or other hoverfly species with an aquatic larval stage were amplified. When aliquots of *E. cryptarum* DNA were mixed with *E. horticola* DNA at different ratios, this specific primer set was successful at amplifying *E. cryptarum* in all volumes with no amplification of solely *E. horticola* DNA (Fig. 10). As there was no amplification of *E. horticola* DNA when *E. cryptarum* DNA was absent, the primers are shown to be able to amplify *E. cryptarum* DNA at low quantities and in the presence of another closely related species DNA. In the dilution series, these primers remained effective up to a dilution of 10^{-4} with specimens Ecryptarum1 and Ecryptarum2 also producing positive bands at a 10^{-5} dilution (Fig. 9).

Table 6 Starting DNA concentrations of the specimens used in each dilution series. For each specimen used, further information in regard to collection location can be found in Table 8.

Species	Specimen ID	ng/µl
E. tenax	Etenax1	55.3
E. horticola	Ehorticola1	33.6
E. cryptarum	Ecryptarum1	17.8
E. cryptarum	Ecryptarum2	65.8
E. cryptarum	Ecryptarum3	28.5
E. arbustorum	Earbustorum1	93.3
E. arbustorum	Earbustorum2	89.3

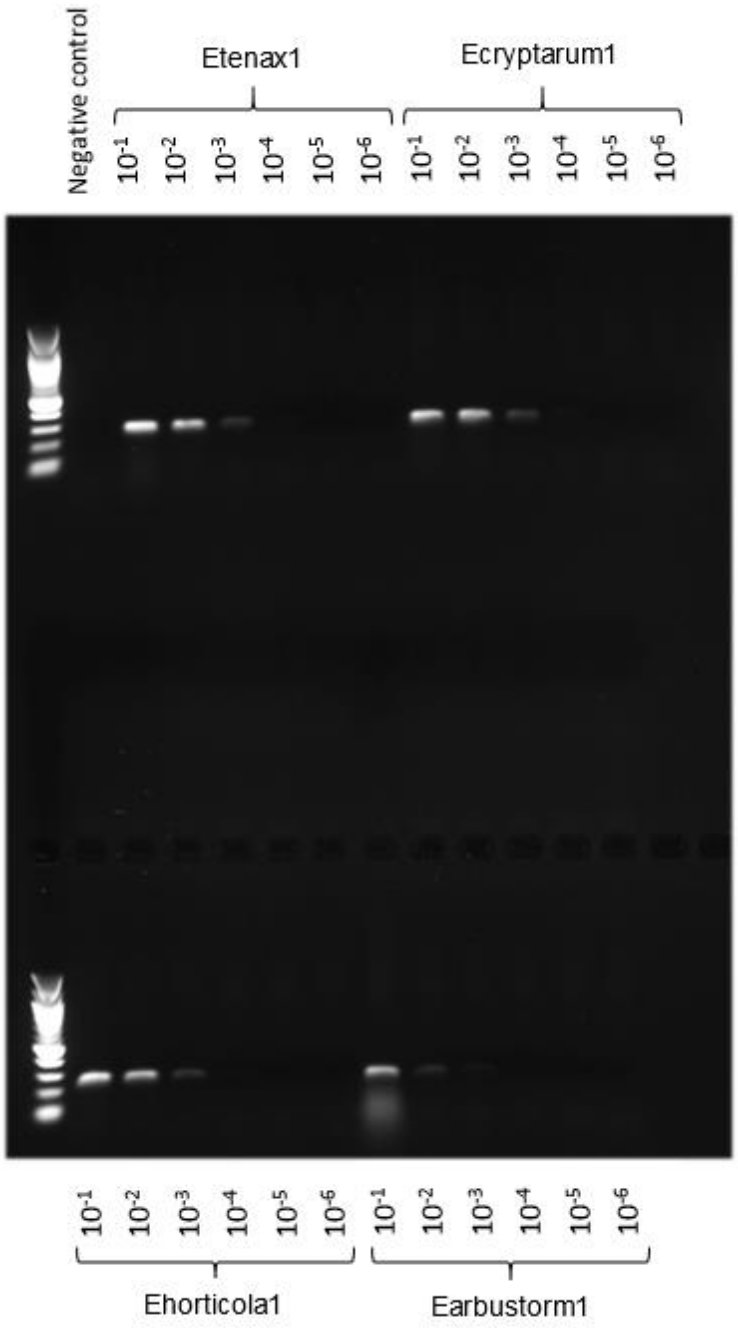


Fig. 7 Dilution series for *Eristalis* species: Etenax1, Ecryptarum1, Ehorticola1 and Earbustorum1 (Table 6) from a 10^{-1} to 10^{-6} dilution. The first blank gap is a negative control using the primer set ErisF2 and ErisR1

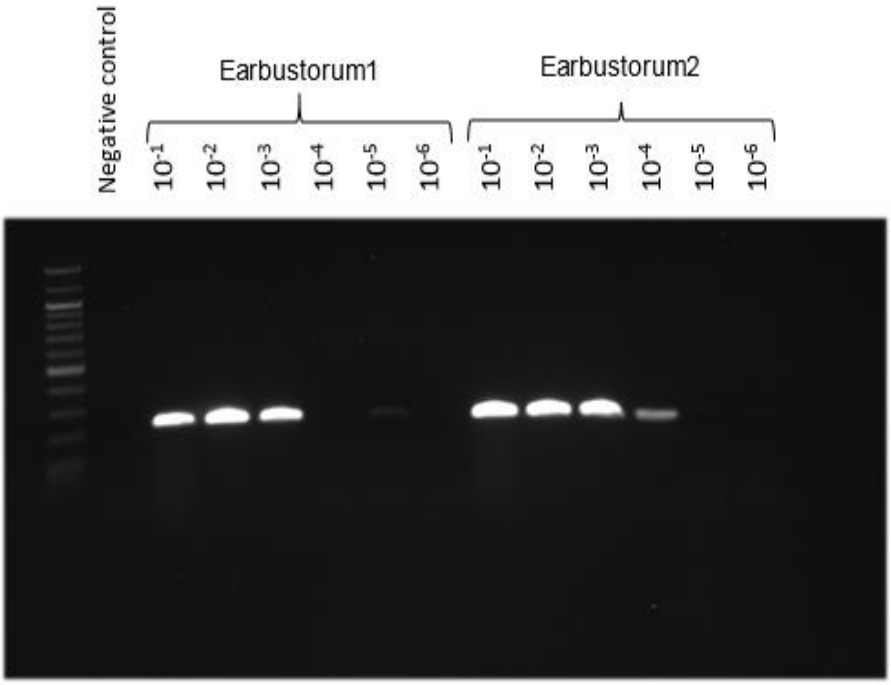


Fig. 8 Dilution series for two specimens of *E. arbustorum:* Earbustorum1 and Earbustorum2 (Table 6) from a 10^{-1} to 10^{-6} dilution using the EarbF1 and EarbR1 primer set. The first blank gap is a negative control.

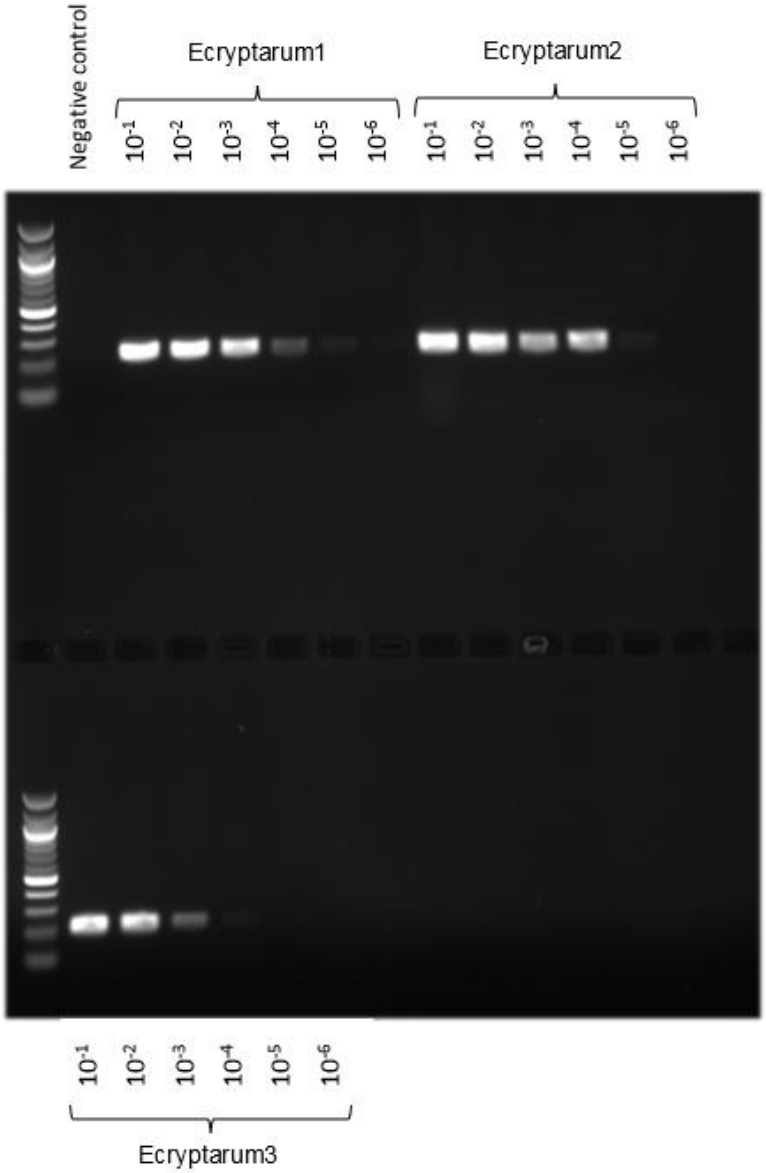


Fig. 9 Dilution series for three specimens of *E. cryptarum:* Ecryptarum1, Ecryptarum2 and Ecryptarum3 (Table 6) from a 10^{-1} to 10^{-6} dilution using the EcrypF3 and EcrypR4 primer set. The first blank gap is a negative control.

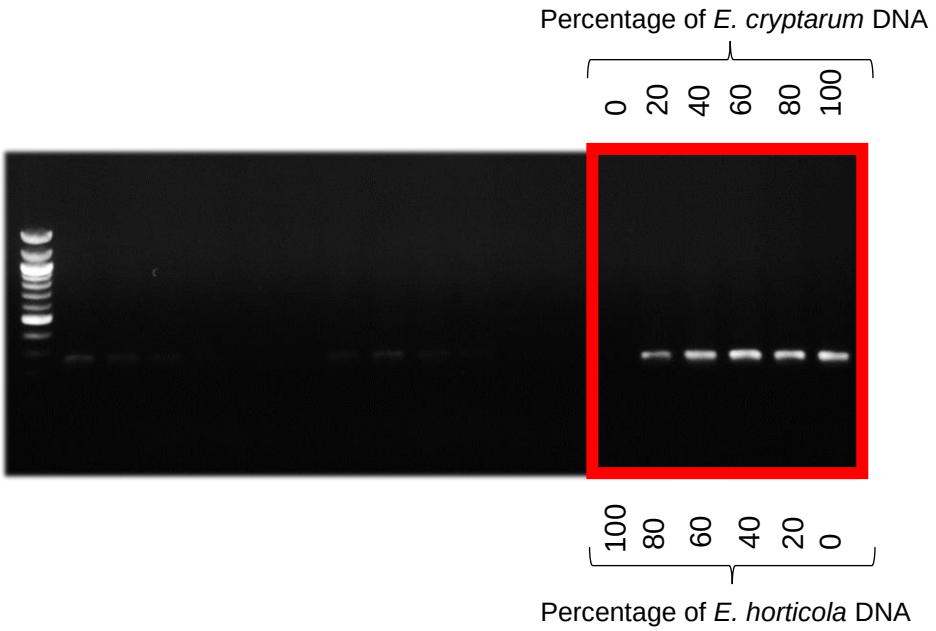


Fig. 10 *E. cryptarum* DNA mixed with *E. horticola* DNA at different ratios (Table 4).

Discussion:

The use of eDNA techniques for the detection of cryptic taxa has been widely applied with considerable success in marine and freshwater environments (e.g. Ficetola, 2008, Olsen, Briggler & Williams, 2012, Pilliod *et al.* 2013, Goldberg *et al.* 2013). However, the development of species-specific primers is key to the success of this molecular tool in order to minimise the potential occurrence of false positive and false negative results in the PCR amplification of template DNA from environmental samples (Macdonald & Sarre, 2017). The main focus of the work carried out here was to develop a primer set specific to the endangered bog hoverfly *E. cryptarum* in preparation for an eDNA study surveying natural water bodies in Dartmoor National Park for the presence of this endangered species of hoverfly. This approach appeared successful, and

the resultant primer set was not only highly specific, but also sensitive and able to work when DNA aliquots were diluted down to a 10^{-4} dilution. In Chapter 4, the presence or absence of *E. cryptarum* eDNA will be determined in water samples taken from across the moor. And so, the amplification of non-target species DNA or, alternatively, the inability of primers being able to amplify *E. cryptarum* eDNA when it is present can have significant effects on any results obtained, potentially proving misleading. Other *Eristalis* species are commonly found in the same habitat as *E. cryptarum,* including *E. tenax, E. nemorum, E. arbustorum, E, intricarius, E. pertinax* and *E. horitcola* and so the ability of an *E. cryptarum*-specific primer set to avoid amplifying closely related, non-target species is vital. The primer set specific to *E. arbustorum:* EarbF1 and EarbR1 proved to also be highly specific to the target species without amplifying any other aquatic hoverfly species, as well as being able to amplify reliably down to a 10^{-3} dilution.

The ErisF2 and ErisR1 primer set was developed to amplify only species belonging to the *Eristalis* genus; this was partially successful. However, this primer set also amplified two other hoverfly species *Neoascia tenur* and *Rhingia campestris*, the larvae of which are also found in aquatic habitats (Stubbs & Falk, 2002). For the purpose of the eDNA study presented in Chapter 3, this was deemed acceptable for two reasons. Firstly, a positive result using this primer set still demonstrates that hoverfly eDNA can be amplified from environmental samples and indicates the presence of aquatic larvae. Secondly, when using species-specific primer sets to target *E. cryptarum,* a positive result can be further verified if a positive result is also obtained using the ErisF2 and ErisR1 primer set. It must be noted however, that the ErisF2 and ErisR1 primer set was slightly less sensitive in the dilution series when compared to the *E.*

cryptarum and *E. arbustorum* specific primer sets. This may be a potential problem if a positive result is obtained using species-specific primers but not with the ErisF2 and ErisR1 primer set, as it would have to be determined if this was a true positive result but the target eDNA was just too diluted for the ErisF2 and ErisR1 primer set. The non-specificity of primer sets for eDNA studies has been deemed advantageous in certain cases, for example, when eDNA was utilised for the detection of multiple invasive species of carp (Bronnenhuber & Wilson, 2013). Herbert (2003) demonstrated how the *cox1* gene can be used as a DNA barcoding tool for species identification and how use of this gene works particularly well for insects. The *cox1* gene has proven useful in the current study in terms of allowing the targeting of specific species for primer design, however, the genus-wide primer set for *Eristalis* proved more difficult to refine due to a reduction in sequence variation at a higher taxonomic level. But, using mitochondrial genes for eDNA studies has additional advantages, such as the DNA being protected by the mitochondria from environmental factors including, temperature or UV exposure (Turner *et al.* 2014, Wilcox *et al.* 2013). The success in development of these taxon-specific primer sets has been demonstrated using high-quality DNA extracts, which has provided a robust framework in which to assess the ability of these primers to amplify potentially degraded target-species DNA in environmental samples. The results of this are presented in Chapter 4.

Macdonald & Sarre (2017) developed a framework for the development and validation of species-specific primers to use in eDNA studies. They discussed the need for a standardised method to test the sensitivity and specificity of primers used for the detection of a target species in environmental samples; frequently, however, this is not performed in eDNA studies. Some past studies

appear to have given little attention to primer design and these have then been used for eDNA analysis without any further testing of the specificity and sensitivity of these primers (Goldberg *et al.* 2011, Roussel *et al.* 2015). Understanding the sensitivity of primers is critical to studies with potential orders of magnitude difference in concentration and quality of target DNA. If the primer set used has low sensitivity then the probability of correct amplification of target DNA is reduced, and thus the occurrence of false negative results is potentially increased (Macdonald & Sarre, 2017). Roussel *et al.* (2015) provide a very critical perspective of using eDNA as a survey tool for aquatic species. They voice their concerns in regard to the inconsistency in eDNA studies, of which some of these inconsistencies include the absence of information regarding primer sensitivity. The results obtained from eDNA studies can be interpreted in many ways and there are many factors to consider that may affect eDNA detection rates, such as DNA degradation or the design of reliable primer sets (Roussel *et al.* 2015; Sassoubre *et al.* 2016; Macdonald & Sarre, 2017; Goldberg, Strickler & Fremier, 2018). And so, by establishing the robustness of the primers used prior to the analysis of environmental samples, any results obtained with field samples (Chapter 4) can be interpreted with more confidence and with a better ability to distinguish false positive and negative results.

Chapter 4

Development of a species-specific eDNA methodology: the search for the Bog hoverfly *Eristalis cryptarum* (Syrphidae, Diptera)

Abstract:

The critically endangered Bog hoverfly *Eristalis cryptarum* is a UK Biodiversity Action Plan priority species and is confined to a number of sites on Dartmoor in southwest England. The larvae of this species have never been found in the UK but are presumed to be aquatic (Chapter 2). In order to target conservation efforts effectively, it would be beneficial to know specific larval habitat requirements and distribution. The aim of this study is to develop a method that will determine the presence or absence of *E. cryptarum,* in order to accurately map its distribution across Dartmoor. To do this, an eDNA methodology is developed to facilitate species-specific rapid screening for *E. cryptarum* DNA in water samples. 'Free' or environmental DNA (eDNA) can be released by, for example, faeces, urine or sperm and one sample may contain the DNA of multiple organisms. Water samples were collected from known *E. cryptarum* sites across Dartmoor. Sites for aquatic sampling will be identified on the basis of positive identification of adult flies during field visits. Specific primer sets designed and tested in Chapter 3 are used here to screen for *E. cryptarum* and other hoverfly eDNA in these water samples, by targeting a fragment of the mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) gene. *E. arbustorum* larvae were kept in the laboratory in a known volume of water throughout this project and water samples were continuously collected from this. *E. arbustorum* DNA is alsotargeted using an *E. arbustorum*-specific primer set to further test

this methodology. A successful amplification of *E. arbustorum* DNA was obtained for one water sample. This demonstrates the potential of an eDNA technique for a hoverfly species. There was no DNA amplification from water samples collected from Dartmoor. Further research needs to be carried out in order to further optimise this method, preferably on water samples that are known to have contained *E. cryptarum* larvae in order to distinguish between true and false negative results.

Introduction:

The surveying of an endangered species is a vital part of their conservation. But when a species is rare or extremely elusive, traditional surveying methods alone may not paint the whole picture in terms of species distribution. With traditional sampling tools can come increased sampling effort, cost, and in some cases, lower detection rates (Biggs *et al.* 2015; Smart *et al.* 2015). In addition, traditional surveying methods can be stressful for the animal or sampling and handling may be restricted for some rare or endangered species (Smart *et al.* 2015). The screening of environmental DNA (eDNA) from environmental samples is a method that has become increasingly popular and seen as a highly beneficial monitoring technique (Pilliod *et al.* 2013; Bohmann *et al.* 2014; Rees *et al.* 2014; Ficetola *et al.* 2015). Environmental DNA is 'free' DNA that is released in to the environment by a variety of sources, for example: blood, pollen or faeces (Bohmann *et al.* 2014) and can be extracted from a number of different types of environmental sample, including but not restricted to: ice cores, soil, water and cave sediments (Taberlet *et al.* 2012; Bohmann *et al.* 2014). Using an eDNA approach has been widely applied to ecological studies such as: biodiversity assessments (Calvignac-Spencer *et al.* 2013), diet analysis (Guillerault *et al.* 2017), disease detection (Walker *et al.* 2007) and the reconstruction of past flora and fauna (Willerslev, *et al.* 2003). As well as this, the application of eDNA as a survey tool has been utilised to monitor species, for instance for the detection of invasive species such as the American bullfrog *Rana catesbeiana* (Ficetola *et al.* 2008) or New Zealand mudsnails *Potamopyrgus antipodarum* (Goldberg *et al.* 2013). The same has been done for other species that are particularly rare, for instance, the eDNA approach was

successfully applied to the monitoring of the endangered Macquarie perch *Macquaria australascia* (Piggott, 2016).

The bog hoverfly *Eristalis cryptarum* has always been known for being a particularly rare and elusive hoverfly. *E. cryptarum* is now critically endangered and listed as a priority species on the UK Biodiversity Action Plan (UK Biodiversity Group, 1999) and is restricted to a number of sites on Dartmoor National Park, Devon, UK. Past and current efforts that have focused on the surveying of *E. cryptarum* have largely been down to the efforts of local naturalists, where long hours of hard work and searching in the field usually results in a small number of records (Ramel 1998; Perret, 2001; Drake and Baldock, 2002; Drake and Baldock, 2003; Drake and Baldock, 2004). This previous work has highlighted the difficulty in surveying this species, and how extremely elusive *E. cryptarum* is. The larvae are widely presumed to be aquatic and previous work in Chapter 2 exploring the phylogeny of *E. cryptarum* has contributed to the support of this assumption. Species specific primer sets have already been successfully developed for *E. cryptarum* and generic hoverfly species with an aquatic larval stage in Chapter 3 in preparation for this study. Here, is the first attempt to develop an eDNA screening tool to detect the presence or absence of bog hoverfly *Eristalis cryptarum* larval eDNA from water samples collected from Dartmoor, in order to contribute to the monitoring of this endangered and elusive species. Alongside this, *E. arbustorum* larvae housed in the laboratory will act as a model to test the ability of obtaining hoverfly eDNA from water samples using an *E. arbustorum* specific primer set.

Materials and Methods

Water sample collection:

Water samples were collected from known *E. cryptarum* habitat sites on Dartmoor National Park: Corndonford, Pizwell and Buckland Common. This was based on where *E. cryptarum* was sighted regularly during this study between May-October 2017. These are also sites where *E. cryptarum* has been recorded recently in previous years and where habitat quality was judged to still be suitable for this species. At one of the sites known as Corndonford, physical markers were used to mark where individuals were observed guarding territories and basking and GPS recordings were taken. Water samples were collected from both these specific points and across other unmarked locations throughout the site. Where water was sampled from was dependent on the structure of the habitat in terms of the direction of runnels and patches of 'boggy' habitat. The method used for water collection was taken from Ficetola *et al.* (2008). 1.5 ml of sodium acetate 3M and 33 ml of absolute ethanol was added to each 15 ml water sample. Due to the nature of the habitat from where these water samples were being collected and the small amount of water present, this was deemed the most appropriate method to use. During collection the water samples were kept on ice until transported back to the laboratory where they were stored at -20˚C until ready for DNA extraction. A GPS reference was recorded for every water sample collected (Appendix, Table 9).

Table 7 The date and sample ID for every water sample collected from water kept in the laboratory housing *E. arbustorum* larvae. The number of live *E. arbustorum* present at time of collection is given.

Date	Sample ID	Number of live larvae present
16/10/2017	A	32
16/10/2017	B	32
19/10/2017	C	14
19/10/2017	D	14
11/11/2017	E	13
11/11/2017	F	13
13/11/2017	G	13
13/11/2017	H	13
23/11/2017	I	12
23/11/2017	J	12
30/11/2017	K	12
30/11/2017	L	12
07/12/2017	M	12
07/12/2017	N	12
14/12/2017	O	12
14/12/2017	P	12
20/02/2018	Q	10
20/02/2018	R	10
14/03/2018	S	7
14/03/2018	T	7
06/06/2018	U	0
06/06/2018	V	0

E. arbustorum larvae were collected and housed in the laboratory in 50 ml of water as described in Chapter 3. Water samples were collected from this using the same method as described above. Once these water samples were collected and stored at -20 ˚C, water taken from a stream on the University of Exeter campus was used to refill the pot housing *E. arbustorum* larvae. The date for each water sample collection and the number of larvae present was recorded (Table 7). Water samples were continuously collected until the death or pupation of the larvae.

DNA analysis:

In order to isolate any environmental DNA present, the water samples were centrifuged (3184g, 45 min, 6˚C) (adapted from Ficetola *et al.* 2008) and the supernatant was discarded (Valiere & Taberlet, 2000). DNA extraction was then performed on the remaining pellet using QIAGEN DNeasy® Blood and Tissue Kit (Qiagen GmbH, Germany) according to the manufacturer's instructions. If there was a large resultant pellet, three DNA extractions were performed per pellet. The nucleic acid concentration and purity of the resulting products was measured using NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). PCR reactions varied slightly depending on the primers used. DNA extracts from all collected water samples were screened with the ErisF2 and ErisR1 primer set and PCR reactions were performed in 10 µl volumes: 5 µl HotStart Taq Master Mix (Qiagen), 3.5 µl RNase free water, 0.2 µl of each primer, 0.1 µl BSA and 1 µl DNA extract. PCR was performed as follows: an initial denaturing temperature of 94˚C for 5 minutes, 35 cycles of 30 seconds denaturing at 94˚C, 30 seconds annealing at 50˚C and a 1 minute extension at 72˚C followed by a final extension for 7 minutes at 72˚C. DNA extracts from water samples collected from *E. cryptarum* habitat sites on Dartmoor were further screened with the *E. cryptarum* specific primer sets EcrypF3 and EcrypR4. PCR reactions were performed in 10 µl volumes as described above. PCR was performed as follows: an initial denaturing temperature of 94˚C for 5 minutes, 35 cycles of 30 seconds denaturing at 94˚C, 30 seconds annealing at 57˚C and a 1 minute extension at 72˚C followed by a final extension for 7 minutes at 72˚C. Finally, DNA extracts from *E. arbustorum* larvae water samples collected from the laboratory were screened using the *E. arbustorum* specific primer set EarbF1 and EarbR1. PCR reactions were

performed in 10 µl volumes as described above. PCR was performed as follows: an initial denaturing temperature of 94˚C for 5 minutes, 35 cycles of 30 seconds denaturing at 94˚C, 30 seconds annealing at 55˚C and a 1 minute extension at 72˚C followed by a final extension for 7 minutes at 72˚C. For each DNA extract, three PCR reaction replicates were performed. For every PCR performed, negative controls were included. The amplified PCR product was separated using gel electrophoresis on a 1% agarose gel with a negative and positive control. A positive band at the appropriate length (dependent on the primer set used, see Table 5) indicates the presence of targeted eDNA.

Results:

E. arbustorum water samples

In total, 22 water samples were collected from where *E. arbustorum* larvae were kept between October 2017 and June 2018. All water samples underwent DNA extractions and for each water sample, three DNA extractions were performed. Three PCR reactions were performed for each DNA extract using only the EarbusF1 and EarbusR2 species specific primer set. Out of these, successful amplification for all replicates for one water sample were obtained. A further three PCR replicates were performed on each DNA extract using the ErisF2 and ErisR1 primer set and no positive results were obtained.

Dartmoor water samples

35 water samples collected from Dartmoor were selected and underwent DNA extraction. Three PCR replicates were performed for each DNA extract using the EcrypF3 and EcrypR4 primer set, and a further three PCR replicates per

DNA extract using the ErisF1 and ErisR1 primer set. Of all these, there was no amplification of the target *cox1* gene fragment and therefore no positive results.

Discussion:

The use of eDNA as a tool to survey rare and endangered species has been successfully applied to a variety of taxa, such as: fish (Takahara *et al.* 2012; Thomsen *et al.* 2012), amphibians (Ficetola *et al.* 2008; Biggs *et al.* 2015), mammals (Schnell *et al.* 2012; Thomsen *et al.* 2012; Calvignac-Spencer *et al.* 2013) and invertebrates (Goldberg *et al* 2013; Tréguier *et al.* 2014). Applying an environmental DNA tool for the surveying of insects, however is less represented (Roussel *et al.* 2015). Here, this approach has been developed to specifically survey the endangered bog hoverfly *E. cryptarum* for the first time. Keeping *E. arbustorum* larvae in the laboratory allowed the screening of water samples (using species specific primer set developed in Chapter two) which held a known number of larvae. Being able to test species-specific primers on water samples where hoverfly larvae were known to be present was an important step in the validation of using an eDNA technique to survey *E. cryptarum.* However, *E. arbustorum* DNA was only successfully amplified in one sample out of a total of 22 water samples. Considering larvae were kept in this water at all times, this lone positive result demonstrates a great deal about using eDNA for hoverflies and indicates that amplifying hoverfly DNA from environmental samples may not plausible. This could be due to several reasons. First, the amount of DNA shed by hoverfly larvae is unknown. Determining the amount of DNA shed by an organism is rarely done in eDNA studies (Sassoubre *et al.* 2016), and the few studies that have explored this are

generally focused on larger species, such as fish (Klymus, *et al.* 2014; Sassoubre *et al.* 2016). Understanding this, combined with the knowledge of the sensitivity of the primers used determined in Chapter 2 would demonstrate the full sensitivity of an eDNA approach to surveying *E. cryptarum.* Secondly, eDNA is subjected to a vast array of environmental conditions, including: UV exposure, temperature fluctuations, and differing pH levels dependent on the habitat type (Strickler, Fremier & Goldberg, 2015; Sassoubre *et al.* 2016; Goldberg, Strickler & Fremier, 2018). Strickler, Fremier & Goldberg (2015) used bullfrog tadpoles *Lithobates catesbeianus* as a study species to determine the effects of UV-B radiation, pH and temperature on the rate of eDNA degradation. They found that the rate of eDNA degradation increased with higher temperatures, increased UV-B radiation and neutral or acidic pH. The habitat specific to *E. cryptarum* are in boggy habitats on Rhôs pastures. Rhôs pastures are by definition wet grasslands that have acidic soils, and so may contribute to an increased rate of eDNA degradation (Perrett, 2001; De Vere, 2007). As previously discussed in Chapter 3, the *cox1* gene is often targeted in eDNA studies due to the mitochondrion offering some protection from the environment. But it must be considered that the environmental conditions typical of *E. cryptarum* habitat may be so extreme that rates of eDNA degradation can still be high.

This study indicates that a surveying approach using eDNA for this species may not be suitable without further optimisation of this technique and the opportunity to test water samples from bodies of water known to be in habited by *E. cryptarum*. The main issue here is the presence of false negatives. Where it was known *E. arbustorum* larvae were present, there was no amplification of DNA, except for one sample, and so perceived as a negative result. However,

as the presence of larvae was already known, this is a false negative result. So, when this method was applied to water samples collected from Dartmoor and negative results were obtained, it is impossible to distinguish whether this is a false negative and *E. cryptarum* eDNA is in fact present, or if *E. cryptarum* is indeed absent. Of course, the occurrence of negative results when screening for *E. cryptarum* may indeed indicate the absence of *E. cryptarum* larvae from collected environmental samples. Some adult hoverfly species do indeed occur far from where the larval stage may occur as discussed in Chapter 2 (Hart, Bale & Fenlon, 1997; Stubbs & Falk, 2002). However, from the results obtained when amplifying *E. arbustorum* eDNA from water where the larvae was known to be present, it is unwise to assume that these negative results when screening for *E. cryptarum* are true negative results. With an eDNA survey tool, the occurrence of false negative (and indeed false positive) results is a frequent problem and is a known drawback of using an eDNA approach to surveying (Roussel *et al.* 2015; Macdonald & Sarre, 2017).

Further aspects of this eDNA methodology need to be tested before it can be concluded that using an eDNA approach to determine the presence or absence of *E. cryptarum* eDNA is viable or not. The amount of DNA a rat-tailed larvae sheds needs to be measured, preferably for an *Eristalis* species similar in size to *E. cryptarum.* Furthermore, *E. cryptarum* DNA (from an adult specimen) or larval DNA released from another *Eristalis* species should be subjected to different pH levels, temperature fluctuations and UV radiation to determine the rate of DNA degradation in water. However, this study suggests that a molecular approach to surveying an elusive species such as *E. cryptarum* requires a level of further optimisation, however testing and re-testing was not possible within the duration of the current research project. Ultimately, whether

the use of such an approach proves viable, will also depends on how much eDNA hoverfly larvae, and in particular the larvae of *E. cryptarum*, shed into their freshwater habitat.

Chapter 5: General discussion

Since its rediscovery after being presumed extinct, *Eristalis cryptarum* has been a species of conservation concern and of great interest to local ecologists and entomologists. Little is known about this species, especially in regard to its larval stage. Understanding the larval stage of *E. cryptarum* would aid in the understanding of this species requirements and would assist in the tailoring of conservation efforts to key larval habitats. The aim of this thesis was to further current understanding in regard to the larval stage of *E. cryptarum.* Following from this, an alternative surveying tool using environmental DNA (eDNA) released by *E. cryptarum* larvae was developed as a potentially quick and non-invasive method to survey a particularly rare and elusive species.

It is widely presumed *E. cryptarum* have an aquatic, rat-tailed larval stage (Stubbs & Falk, 2002) similar to closely related species within the Eristalini tribe. However, with the absence of *E. cryptarum* larvae to confirm this, the phylogeny of *E. cryptarum* was explored using the mitochondrial gene cytochrome *c* oxidase subunit 1 (CO1) and morphological characters in order to explore this assumption in Chapter 2. This study is the first time the phylogeny of *E. cryptarum* has been determined (to the authors knowledge) with the use of molecular data. All other species placed within the Eristalini tribe match previous knowledge in regard to Syrphidae taxonomy and *E. cryptarum* was confidently placed within the *Eristalis* genus (in the Eristalini tribe). A key characteristic shared by the Eristalini tribe is an aquatic, rat-tailed larval stage (Rotheray, 1999; Stubbs & Falk, 2002) and so supports the assumption that *E.*

cryptarum has an aquatic larval stage. Using the *cox1* gene worked well in this instance, especially for the Eristalini tribe. However, outside of the Eristalini tribe there are areas of low resolution between other Syrphidae tribes, potentially due to the time of divergence within the Syrphidae family (Wiegman *et al.* 2011). Alternative genes could be used in addition to the *cox1* gene to help clarify this, such as the 28S rRNA gene (Ståhls *et al*. 2003). Nonetheless, here the purpose was to confirm the placement of *E. cryptarum* which was successfully achieved. For an endangered species such as *E. cryptarum*, having more of an understanding of all life stages allows the tailoring of effective conservation efforts (New, 2007; Rotheray, Goulson & Bussière, 2016). For the purpose of this thesis, the results from Chapter 1 have supported the assumption *E. cryptarum* may have an aquatic, rat-tailed larval stage and so provides a foundation for the development of an eDNA methodology to survey for *E. cryptarum* in the second part of this thesis (Chapter 3 and 4).

In the second part of this thesis, an alternative surveying tool was explored using eDNA. *E. cryptarum* is a notoriously difficult species to survey using traditional techniques (Drake & Baldock, 2005). The authors own experience during field visits as well the accounts of others who have surveyed *E. cryptarum* demonstrate that many hours need to be spent surveying in order to gain few records (Ramel, 1998; Perret, 2001; Drake & Baldock, 2002; Drake & Baldock, 2003; Drake & Baldock, 2004). Utilising eDNA as an alternative surveying method has been developed and applied successfully to a number of other species such as: fish (Takahara *et al.* 2012; Thomsen *et al.* 2012), amphibians (Ficetola *et al.* 2008; Biggs *et al.* 2015), mammals (Calvignac-Spencer *et al.* 2013; Schnell *et al.* 2012) and invertebrates (Goldberg *et al*

2013; Tréguier *et al.* 2014). But this has never been attempted for *E. cryptarum* before. Chapter 3 was focused on the development of taxa-specific primers that would be used for further application of the eDNA methodology in Chapter 4. Here, primer sets were successfully developed to amplify a fragment of the *cox1* gene for the *Eristalis* genus, *E. cryptarum* and a closely related species *E. arbustorum.* Species-specific primer sets developed for *E. arbustorum* and *E. cryptarum* were specific to their target species and did not amplify any other species. After testing, the primer sets developed to target the whole *Eristalis* genus also amplified other non-target species. However, as these species also possess an aquatic larval stage this was deemed acceptable for the purpose of eDNA analysis as the ability to amplify hoverfly eDNA is demonstrated and offers further validation of positive results using species-specific primer sets. The sensitivity of the primer sets was also tested through a number of dilutions and showed that these primer sets were able to still work well with heavily diluted DNA aliquots. Determining the specificity and sensitivity of each primer set is extremely important as an environmental sample will hold the DNA of hundreds of organisms as well as being diluted depending on an organism's DNA shedding rate or the rate of environmental degradation (Bohmann *et al.* 2014; Sassoubre *et al.* 2016; Macdonald & Sarre, 2017). And so, assuring the primers are specific to the target taxa limits the possibility of obtaining a false positive result while screening environmental samples, therefore reduces the possibility of misleading data (Macdonald & Sarre, 2017).

In Chapter 4, the primer sets developed in Chapter 3 were used to screen water samples for hoverfly eDNA with the aim of developing a methodology to survey *E. cryptarum* by molecular means. As the larvae of a closely related species, *E.*

arbustorum were able to be kept in the laboratory, water samples could be collected from this with the knowledge that larvae are definitely present. From these water samples, successful amplification of *E. arbustorum* eDNA was obtained for 1 water sample out of 22. Being able to amplify *E. arbustorum* eDNA in one water sample demonstrates that this method has the potential to be effective but as *E. arbustorum* was known to be present in all samples, there is an occurrence of false negative results and further optimisation of this method is needed. No *E. cryptarum* eDNA was successfully amplified from environmental water samples, but as claimed previously, whether or not these are true negative results needs further testing. Future research regarding the optimisation of this method would need to include quantification of DNA shedding rates of *Eristalis* larvae. The case may be that larvae simply do not shed enough DNA to be detected, despite the sensitivity of an eDNA technique (Bohmann *et al.* 2014; Smart *et al.* 2015). Environmental conditions also need to be considered as acidity, temperature and UV radiation can all influence eDNA degradation rates (Strickler, Fremier & Goldberg, 2015; Sassoubre *et al.* 2016; Goldberg, Strickler & Fremier, 2018). And so, the environmental conditions of the habitat specific to *E. cryptarum* need to be measured and replicated in order to investigate the effects of these specific environmental conditions on hoverfly and in particular, *E. cryptarum* eDNA.

Appendix

Table 8 List of all 101 specimens collected throughout this project including their site of collection and grid reference. Collection ID provides the initials of the individual who collected the specimen, CM (Catherine Mitson), JW (John Walters) and DFG (Devon Fly Group). Specimens in blue are those that were used in Chapter 2 and numbers given in square brackets refer to which specimens are shown in Fig 10, Fig 11 and Table 3. Those in bold are the specimens used in dilution series in Chapter 3 with their alternative ID name used in Chapter 3 in brackets.

Species	Site of collection	Grid reference	Collection ID	Date
Anasymia lineata	Corndonford	SX692748	JW	05/08/2017
Cheilosia tarsus	Buckland	SX734747		01/06/2017
Chrysotoxum bicinctum	Pizwell	SX670786	JW	05/07/2016
Episyrphus balteatus	Buckland	SX734744	CM	22/09/2016
Episyrphus balteatus	Exwick Cemetery Park	SX900928	CM	01/10/2016
Episyrphus balteatus	Pizwell	SX670786	CM	22/09/2016
Episyrphus balteatus	Streatham Campus	SX916940	CM	28/09/2016
Episyrphus balteatus	Streatham Campus	SX916940	CM	28/09/2016
Episyrphus balteatus	Streatham Campus	SX916940	CM	30/09/2016
Eristalinus aeneus	Coleton Fishacre	SX910503	DFG	28/04/2017
Eristalis abusivus	St Davids, The Bug Farm	SM780261	JW	19/08/2017

Table 8 continued

Species	Site of collection	Grid reference	Collection ID	Date
Eristalis arbustorum (larvae)	Gara Rock	SX753369	CM	10/07/2017
Eristalis arbustorum **(Earbustorum2)**	Exwick Cemetery Park	SX900928	CM	01/10/2016
Eristalis cryptarum	Corndonford	SX692746	CM	01/07/2017
Eristalis cryptarum **(Ecryptarum1)**	Corndonford	SX692747	JW	09/07/2018
Eristalis cryptarum **(Ecryptarum2)**	Corndonford	SX692748	CM	15/08/2016
Eristalis horticola [1]	Buckland	SX734744	JW	05/07/2016
Eristalis horticola [2]	Buckland	SX734744	JW	05/07/2016
***Eristalis horticola* [3]** **(Ehorticola1)**	Buckland	SX734744	JW	05/07/2016
Eristalis horticola [4]	Buckland	SX734744	CM	01/06/2017
Eristalis horticola	Buckland	SX734745	CM	01/06/2017
Eristalis intricarus	Corndonford	SX693751	JW	05/09/2017
Eristalis intricarus	Pizwell	SX670786	CM	22/09/2016
Eristalis nemorum	Exminster	SX962871	JW	17/08/2017
Eristalis nemorum [3]	Exwick Cemetery Park	SX900928	CM	01/10/2016
Eristalis nemorum [1]	Moortown Bottom	SX661888	JW	05/07/2016
Eristalis nemorum [2]	Moortown Bottom	SX661888	JW	05/07/2016
Eristalis pertinax	Corndonford	SX693751	JW	11/09/2017

Table 8 continued

Species	Site of collection	Grid reference	Collection ID	Date
Eristalis pertinax	Corndonford	SX693751	JW	11/09/2017
Eristalis pertinax	Corndonford	SX693751	JW	11/09/2017
Eristalis pertinax	Dartmeet	SX672731	CM	07/04/2017
Eristalis pertinax	Exwick	SX902930	CM	01/10/2016
Eristalis tenax	Buckland	SX734744	CM	22/09/2016
Eristalis tenax	Buckland	SX734744	CM	22/09/2016
Eristalis tenax	Challacombe	SX693792	CM	22/09/2016
Eristalis tenax	Exwick Cemetery Park	SX900928	CM	01/10/2016
Eristalis tenax [1]	Pizwell	SX670786	CM	22/09/2016
Eristalis tenax	Pizwell	SX670786	CM	22/09/2016
***Eristalis tenax* (Etenax1)**	Pizwell	SX670786	CM	22/09/2016
Eristalis tenax	Pizwell	SX670786	CM	22/09/2016
Eristalis tenax	Pizwell	SX670786	JW	05/07/2016
Eristalis tenax [3]	Pizwell	SX670786	CM	22/09/2016
Eristalis tenax [4]	Pizwell	SX670786	CM	22/09/2016
Eristalis tenax	Pizwell	SX670786	CM	01/06/2017
Eristalis tenax	Pizwell	SX670786	CM	01/06/2017
Eupeodes latifasciatus	Exwick Cemetery Park	SX900928	CM	01/10/2016
Eupeodes latifasciatus	Streatham Campus	SX916940	CM	30/09/2016
Eupeodes luniger	Streatham Campus	SX916940	CM	28/09/2016
Ferdinandea cuprea	Exwick Cemetery Park	SX900928	CM	01/10/2016
Helophilus pendulus	Haytor	SX764770		16/06/2017
Helophilus pendulus	Pizwell	SX670786	CM	22/09/2016

Table 8 continued

Species	Site of collection	Grid reference	Collection ID	Date
Helophilus pendulus [1]	Pizwell	SX670786	JW	05/07/2016
Helophilus pendulus	Pizwell	SX670786	CM	22/09/2016
Helophilus pendulus [2]	Streatham Campus	SX916940	CM	28/09/2016
Helophilus pendulus	Streatham Campus	SX916940	CM	30/09/2016
Helophilus pendulus	Streatham Campus	SX916940	CM	30/09/2016
Melangyna lasiophthalma	Haytor	SX764770	CM	16/06/2017
Melanostoma mellinum	Pizwell	SX670787	CM	01/06/2017
Melanostoma scalare	Challacombe	SX693799	CM	09/06/2017
Melanostoma sp.	Challacombe	SX693795	CM	26/05/2017
Melanostoma sp.	Challacombe	SX693796	CM	26/05/2017
Melanostoma sp.	Challacombe	SX693796	CM	26/05/2017
Melanostoma sp.	Challacombe	SX693796	CM	26/05/2017
Melanostoma mellinum [1]	Exwick Cemetery Park	SX900928	CM	01/10/2016
Melanostoma mellinum	Streatham Campus	SX916940	CM	28/09/2016
Melanostoma mellinum [2]	Streatham Campus	SX916940	CM	28/09/2016
Melanostoma mellinum	Streatham Campus	SX916940	CM	30/09/2016
Melanostoma scalare	Pizwell	SX670786	CM	22/09/2016
Melanostoma scalare	Pizwell	SX670786	CM	22/09/2016
Melanostoma scalare	Streatham Campus	SX916940	CM	28/09/2016

Table 8 continued

Species	Site of collection	Grid reference	Collection ID	Date
Melanostoma scalare	Streatham Campus	SX916940	CM	28/09/2016
Melanostoma scalare	Streatham Campus	SX916940	CM	30/09/2016
Microdon mutablis	Pizwell	SX670786	JW	05/07/2016
Microdon sp.	Buckland	SX734746	CM	01/06/2017
Microdon sp.	Pizwell	SX670786	CM	01/06/2017
Neoascia tenur	Pizwell	SX670787	CM	26/05/2017
Neoascia tenur	Pizwell	SX670788	CM	26/05/2017
Neoascia tenur	Pizwell	SX670789	CM	26/05/2017
Platycheirus albiminus	Exwick Cemetery Park	SX900928	CM	01/10/2016
Platycheirus albiminus	Streatham Campus	SX916940	CM	30/09/2016
Platycheirus rosarum	Pizwell	SX670788	CM	01/06/2017
Portevinia maculata	Coleton Fishacre	SX910503	DFG	28/04/2018
Rhingia campestris [3]	Buckland	SX734744	CM	22/09/2016
Rhingia campestris	Buckland	SX734744	CM	22/09/2016
Rhingia campestris	Pizwell	SX670786	CM	22/09/2016
Rhingia campestris [2]	Pizwell	SX670786	CM	22/09/2016
Rhingia campestris	Pizwell	SX670786	JW	05/07/2016
Sericomyia silentis	Challacombe	SX693798	CM	09/06/2017
Sericomyia silentis	Pizwell	SX670786	CM	22/09/2016
Sericomyia silentis	Pizwell	SX670786	JW	05/07/2016
Sericomyia silentis	Pizwell	SX670786	JW	05/07/2016

Table 8 continued

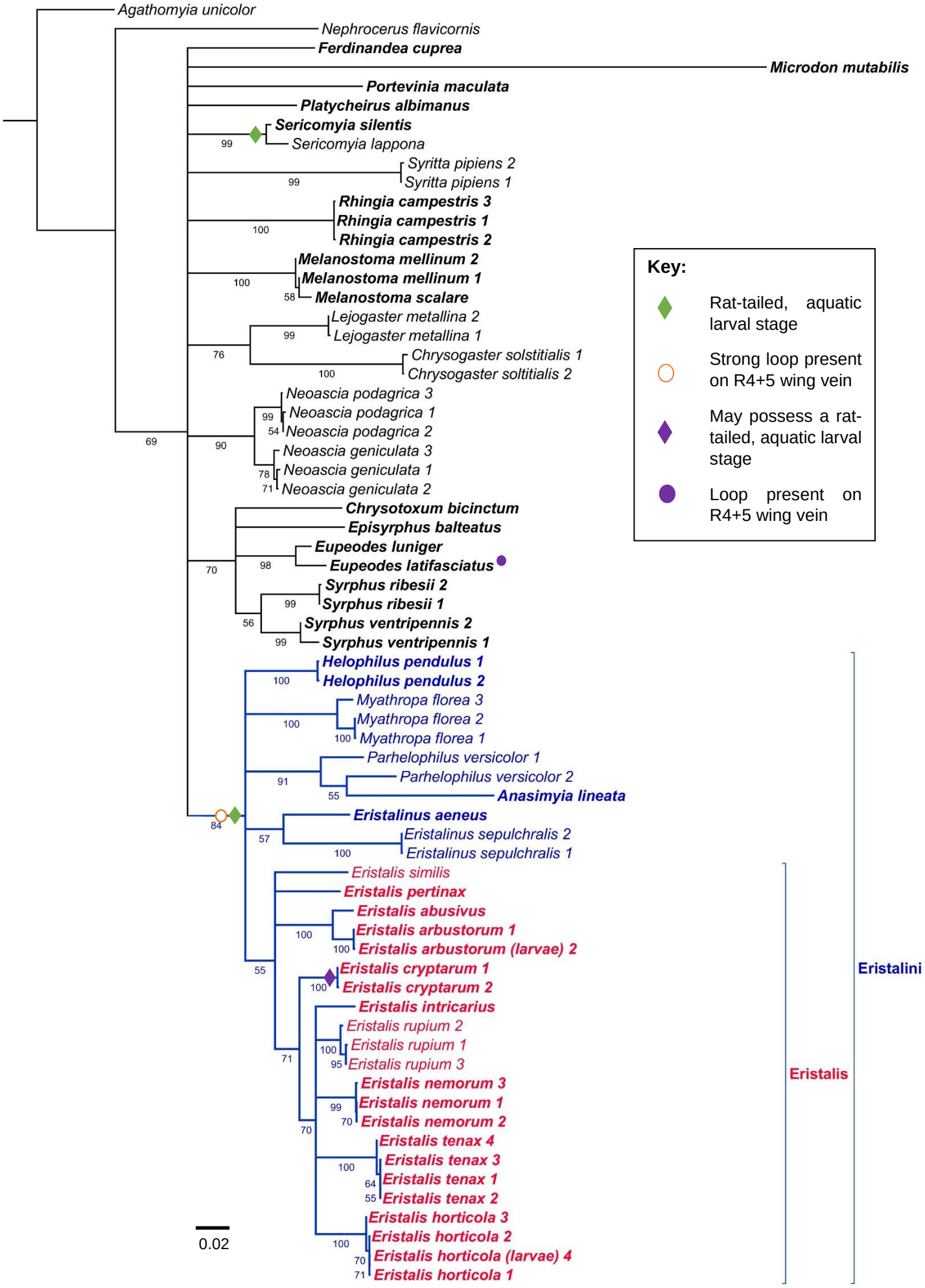
Species	Site of collection	Grid reference	Collection ID	Date
Syrphus ribesii	Exwick Cemetery Park	SX900928	CM	01/10/2016
Syrphus ribesii [1]	Exwick Cemetery Park	SX900928	CM	01/10/2016
Syrphus ribesii [2]	Streatham Campus	SX916940	CM	30/09/2016
Syrphus ribesii	Streatham Campus	SX916940	CM	30/09/2016
Syrphus vetripennis	Haytor	SX764770	CM	16/06/2017
Syrphus vetripennis [1]	Streatham Campus	SX916940	CM	28/09/2016
Syrphus vetripennis [2]	Streatham Campus	SX916940	CM	28/09/2016
Vollucella bombylans	Challacombe	SX693796	CM	09/06/2017
Vollucella bombylans	Challacombe	SX693797	CM	09/06/2017
Xanthogramma citrofasciatum	Pizwell	SX670786	CM	26/05/2017

Table 9 The sample ID, date of collection and location is given for every water sample that was collected from *E. cryptarum* habitat sites on Dartmoor National Park and used in eDNA analysis in Chapter 3.

Sample	Location	Latitude	Longitude	Grid Ref	Date
1	Corndonford Mire	50.559015	-3.8468430	SX69267489	23.08.2017
2	Corndonford Mire	50.559015	-3.8468430	SX69267489	23.08.2017
3	Corndonford Mire	50.559015	-3.8468430	SX69267489	23.08.2017
4	Corndonford Mire	50.559015	-3.8468430	SX69287489	23.08.2017
5	Corndonford Mire	50.559280	-3.8471357	SX69267491	23.08.2017
6	Corndonford Mire	50.559280	-3.8471357	SX69267491	23.08.2017
7	Corndonford Mire	50.559280	-3.8471357	SX69267491	23.08.2017
8	Corndonford Mire	50.559015	-3.8468430	SX69287488	23.08.2017
9	Corndonford Mire	50.559109	-3.8465643	SX69307489	23.08.2017
10	Corndonford Mire	50.559113	-3.8462821	SX69327489	23.08.2017
11	Corndonford Mire	50.559015	-3.8468430	SX69287488	23.08.2017
12	Corndonford Mire	50.558554	-3.8475309	SX69237483	23.08.2017
14	Corndonford Mire	50.559192	-3.8469911	SX69277490	11.09.2017
15	Corndonford Mire	50.561367	-3.8459466	SX69357514	11.09.2017
16	Corndonford Mire	50.561369	-3.8458055	SX69367514	11.09.2017
19	Corndonford Mire	50.561639	-3.8458160	SX69367517	11.09.2017
22	Corndonford Mire	50.561459	-3.8458090	SX69387515	11.09.2017
24	Corndonford Mire	50.561284	-3.8455198	SX69387513	11.09.2017
27	Pizwell	50.593018	-3.8801052	SX67027872	12.09.2017
37	Pizwell	50.593025	-3.8796816	SX67057872	12.09.2017
38	Pizwell	50.593025	-3.8796816	SX67057872	12.09.2017
42	Pizwell	50.593028	-3.8795404	SX67067872	12.09.2017
43	Pizwell	50.593120	-3.8794028	SX67077873	12.09.2017

Table 9 continued

Sample	Location	Latitude	Longitude	Grid Ref	Date
46	Pizwell	50.593025	-3.8796816	SX67057872	12.09.2017
51	Pizwell	50.592940	-3.8793957	SX67077871	12.09.2017
52	Pizwell	50.592940	-3.8793957	SX67077871	12.09.2017
146	Corndonford Mire	50.558826	-3.8474004	SX69247486	26.09.2017
147	Corndonford Mire	50.559093	-3.8475520	SX69237489	26.09.2017
207	Haytor	50.579187	-3.7460752	SX76477695	28.09.2017
208	Haytor	50.579187	-3.7460752	SX76477695	28.09.2017
246	Bonehill	50.583705	-3.7920108	SX73237753	1.10.2017
247	Bonehill	50.584067	-3.7918833	SX73247757	1.10.2017
248	Bonehill	50.584150	-3.7923102	SX73217758	1.10.2017
249	Bonehill	50.584321	-3.7928818	SX73177760	1.10.2017
250	Bonehill	50.584231	-3.7928784	SX73177759	1.10.2017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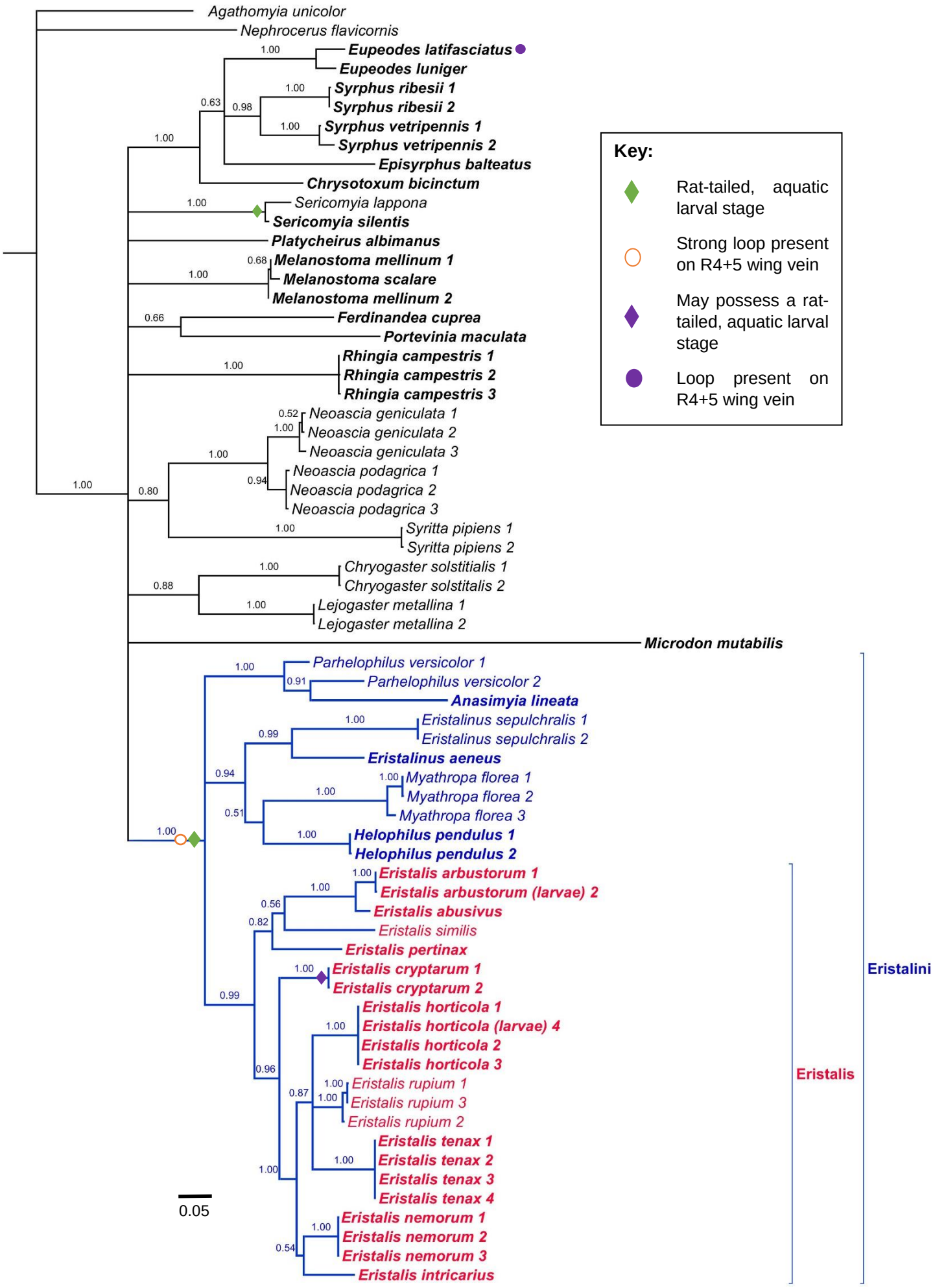
Ferdinandea cuprea
Microdon mutabilis
Portevinia maculata
Platycheirus albimanus
Sericomyia silentis
Sericomyia lappona
Syritta pipiens 2
Syritta pipiens 1
Rhingia campestris 3
Rhingia campestris 1
Rhingia campestris 2
Melanostoma mellinum 2
Melanostoma mellinum 1
Melanostoma scalare
Lejogaster metallina 2
Lejogaster metallina 1
Chrysogaster solstitialis 1
Chrysogaster soltitialis 2
Neoascia podagrica 3
Neoascia podagrica 1
Neoascia podagrica 2
Neoascia geniculata 3
Neoascia geniculata 1
Neoascia geniculata 2
Chrysotoxum bicinctum
Episyrphus balteatus
Eupeodes luniger
Eupeodes latifasciatus
Syrphus ribesii 2
Syrphus ribesii 1
Syrphus ventripennis 2
Syrphus ventripennis 1
Helophilus pendulus 1
Helophilus pendulus 2
Myathropa florea 3
Myathropa florea 2
Myathropa florea 1
Parhelophilus versicolor 1
Parhelophilus versicolor 2
Anasimyia lineata
Eristalinus aeneus
Eristalinus sepulchralis 2
Eristalinus sepulchralis 1
Eristalis similis
Eristalis pertinax
Eristalis abusivus
Eristalis arbustorum 1
Eristalis arbustorum (larvae) 2
Eristalis cryptarum 1
Eristalis cryptarum 2
Eristalis intricarius
Eristalis rupium 2
Eristalis rupium 1
Eristalis rupium 3
Eristalis nemorum 3
Eristalis nemorum 1
Eristalis nemorum 2
Eristalis tenax 4
Eristalis tenax 3
Eristalis tenax 1
Eristalis tenax 2
Eristalis horticola 3
Eristalis horticola 2
Eristalis horticola (larvae) 4
Eristalis horticola 1
0.02
Key:
Rat-tailed, aquatic larval stage
Strong loop present on R4+5 wing vein
May possess a rat-tailed, aquatic larval stage
Loop present on R4+5 wing vein
Eristalini
Eristalis

Fig. 11 Maximum Likelihood tree constructed using a *cox1* dataset. 67 *cox1* sequences were used and here all specimens are displayed. Further information in regard to collection details for each specimen used can be found in Table 3 and additional information for specimens collected throughout this project and used in phylogenetic analysis is given in Table 8 (names given for specimens used in phylogenetic analysis will also be given in Table 8). Specimens in bold are the specimens collected by the author (or donated to) throughout the project and DNA analysis was completed by the author. Values below branches show bootstrap support values (%). Both the Eristalini tribe and *Eristalis* genus is highlighted.○ indicates the presence of a strong loop on the R4+5 wing vein in adults and ● indicates that a loop exists on this vein, but not as evident (specific species possessing this trait are not individually represented,● symbol is instead placed on the relevant tribe next to the species that is most closely related to those species that possess the trait). Species that possess an aquatic, rat-tailed larval stage are indicated by♦and if this is currently unknown, this is shown by♦.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Nephrocerus flavicornis
Eupeodes latifasciatus
Eupeodes luniger
Syrphus ribesii 1
Syrphus ribesii 2
Syrphus vetripennis 1
Syrphus vetripennis 2
Episyrphus balteatus
Chrysotoxum bicinctum
Sericomyia lappona
Sericomyia silentis
Platycheirus albimanus
Melanostoma mellinum 1
Melanostoma scalare
Melanostoma mellinum 2
Ferdinandea cuprea
Portevinia maculata
Rhingia campestris 1
Rhingia campestris 2
Rhingia campestris 3
Neoascia geniculata 1
Neoascia geniculata 2
Neoascia geniculata 3
Neoascia podagrica 1
Neoascia podagrica 2
Neoascia podagrica 3
Syritta pipiens 1
Syritta pipiens 2
Chryogaster solstitialis 1
Chryogaster solstitalis 2
Lejogaster metallina 1
Lejogaster metallina 2
Microdon mutabilis
Parhelophilus versicolor 1
Parhelophilus versicolor 2
Anasimyia lineata
Eristalinus sepulchralis 1
Eristalinus sepulchralis 2
Eristalinus aeneus
Myathropa florea 1
Myathropa florea 2
Myathropa florea 3
Helophilus pendulus 1
Helophilus pendulus 2
Eristalis arbustorum 1
Eristalis arbustorum (larvae) 2
Eristalis abusivus
Eristalis similis
Eristalis pertinax
Eristalis cryptarum 1
Eristalis cryptarum 2
Eristalis horticola 1
Eristalis horticola (larvae) 4
Eristalis horticola 2
Eristalis horticola 3
Eristalis rupium 1
Eristalis rupium 3
Eristalis rupium 2
Eristalis tenax 1
Eristalis tenax 2
Eristalis tenax 3
Eristalis tenax 4
Eristalis nemorum 1
Eristalis nemorum 2
Eristalis nemorum 3
Eristalis intricarius
0.05
Key:
Rat-tailed, aquatic larval stage
Strong loop present on R4+5 wing vein
May possess a rat-tailed, aquatic larval stage
Loop present on R4+5 wing vein
Eristalini
Eristalis

Fig. 12 Bayesian probability tree constructed using a *cox1* dataset. 67 *cox1* sequences were used and here all specimens are displayed. Further information in regard to collection details for each specimen used can be found in Table 3 and additional information for specimens collected throughout this project and used in phylogenetic analysis is given in Table 8 (names given for specimens used in phylogenetic analysis will also be given in Table 8). Specimens in bold are the specimens collected by the author (or donated to) throughout the project and DNA analysis was completed by the author. Values below branches show posterior probability values. Both the Eristalini tribe and *Eristalis* genus is highlighted. ○ indicates the presence of a strong loop on the R4+5 wing vein in adults and ● indicates that a loop exists on this vein, but not as evident (specific species possessing this trait are not individually represented, ● symbol is instead placed on the relevant tribe next to the species most closely to the species that possess this trait). Species that possess an aquatic, rat-tailed larval stage are indicated by ♦ and if this is currently unknown, this is shown by ♦.

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